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Impact of *Lactobacillus plantarum* Sortase on Target Protein Sorting, Gastrointestinal Persistence, and Host Immune Response Modulation

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Sortases are transpeptidases that couple surface proteins to the peptidoglycan of Gram-positive bacteria, and several sortase-dependent proteins (SDPs) have been demonstrated to be crucial for the interactions of pathogenic and nonpathogenic bacteria with their hosts. Here, we studied the role of sortase A (SrtA) in *Lactobacillus plantarum* WCFS1, a model *Lactobacillus* for probiotic organisms. An isogenic *srtA* deletion derivative was constructed which did not show residual SrtA activity. DNA microarray-based transcriptome analysis revealed that the *srtA* deletion had only minor impact on the full-genome transcriptome of *L. plantarum*, while the expression of SDP-encoding genes remained completely unaffected. Mass spectrometry analysis of the bacterial cell surface proteome, which was assessed by trypsinization of intact bacterial cells and by LiCl protein extraction, revealed that SrtA is required for the appropriate subcellular location of specific SDPs and for their covalent coupling to the cell envelope, respectively. We further found that SrtA deficiency did not affect the persistence and/or survival of *L. plantarum* in the gastrointestinal tract of mice. In addition, an *in vitro* immature dendritic cell (iDC) assay revealed that the removal of surface proteins by LiCl strongly affected the proinflammatory signaling properties of the SrtA-deficient strain but not of the wild type, which suggests a role of SDPs in host immune response modulation.

In Gram-positive bacteria, the covalent coupling of proteins to the cell wall peptidoglycan, also known as cell wall sorting, occurs via a conserved mechanism that involves the action of membrane-anchored transpeptidases termed sortases (1). Sortases typically harbor a conserved histidine and a TLXTC motif in their active sites, in which replacement of the single conserved cysteine with alanine has been shown to abolish enzyme activity in *Staphylococcus aureus* (2). Based on primary sequences and substrate specificities, sortases are grouped into four classes (A to D), of which sortase A (SrtA) is referred to as the housekeeping sortase (3, 4). The role of SrtA in surface protein sorting was initially described in *S. aureus* (5, 6), where it was found to recognize proteins bearing the conserved C-terminal cell wall sorting signal LPXTG (7). SrtA cleaves the LPXTG consensus sequence between the threonine and glycine residues and subsequently joins the carboxyl group of threonine to the amino group of the pentaglycine-branched peptidoglycan intermediate lipid II (8). Besides the LPXTG motif, sortase-dependent proteins (SDPs) harbor a C-terminal stop-transfer membrane-anchoring domain (1) that consists of a hydrophobic transmembrane domain followed by positively charged amino acid residues (5, 9, 10), which ensures membrane anchoring of the precursor protein and thereby prevents complete secretion of SDPs prior to peptidoglycan coupling (3).

Several studies focused on the role of sortases in the context of pathogenesis, revealing that sortases assemble virulence factors on bacterial cell surfaces, which is crucial for adhesion and pathogenicity processes (11–16), biofilm formation (17, 18), and iron uptake (19, 20). A limited number of studies focused on sortases in (probiotic) lactobacilli and their role in the interaction with host cells. The deletion of the sortase-encoding gene or the gene encoding the SDP LspA reduced adherence of *Lactobacillus salivarius* UCC118 to epithelial cell lines (21), whereas gastrointestinal (GI) persistence and/or survival of *L. johnsonii* NCC533 in mice was not affected by sortase deletion (22). The Mub protein of *L. acidophilus* NCFM was shown to mediate adhesion to epithelial cell lines and mucin (23), and the pilin protein SpaC of *L. rhamnosus* GG facilitates bacterial binding to host cell mucus (24). The mannose-specific adhesin (Msa) of *L. plantarum* WCFS1 and the genetically closely related strain 299v (25) were identified as key proteins for mannose-specific adhesion, a phenomenon that appeared to be completely sortase dependent (26, 27). In addition, Msa of the latter strain was shown to modulate innate immune response pathways in a small-intestinal-segment perfusion pig model (28). Moreover, *L. plantarum* WCFS1 genes, which were shown to be specifically upregulated during mice GI tract passage, included the genes encoding the SDPs Lp_0800 and Lp_2940 (29, 30), suggesting a particular role for these proteins *in vivo*. This notion was further supported by the finding that an Lp_2940-deficient strain displayed decreased persistence and/or survival in the mouse GI tract (31).

L. plantarum WCFS1 is a model *Lactobacillus* for probiotic organisms, and its genome is predicted to encode a single sortase

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(*srtA*) (32, 33) and 27 SDPs, of which the majority harbor a modified variant of the SrtA consensus sequence, i.e., LPQTXE (34). Here, we studied the role of SrtA in *L. plantarum* WCFS1 and assessed the consequences of *srtA* gene deletion on bacterial cell surface protein sorting by using trypsinization of intact bacterial cells and LiCl extraction of cell surface proteins. Mass spectrometry (MS) analysis of the surface proteome samples derived from the wild type and an SrtA-deficient derivative clearly revealed that SrtA is required for the appropriate subcellular location and for the cell envelope coupling of specific SDPs. In addition, an *in vivo* gastrointestinal survival assay in mice was conducted, demonstrating that *srtA* deficiency did not affect *L. plantarum* GI survival and/or persistence, while an immature dendritic cell (iDC) assay revealed that LiCl-based removal of surface proteins influenced the proinflammatory signaling properties of the SrtA-deficient but not of the wild-type strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains, plasmids, and primers that were used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* strains MC1061 (35) and TOP10 (Invitrogen, Carlsbad, CA) that were used as intermediate cloning hosts for the construction of pNZ7111 and pNZ7102 or pNZ7114, respectively, were grown aerobically in TY medium (36). *L. plantarum* WCFS1 (32), WCFS1-R (29), NZ7104 (27), NZ7102, and NZ7114 (see below) were cultivated without agitation in Mann-Rogosa Sharpe (MRS; Merck, Darmstadt, Germany) medium or 2-times-concentrated chemical defined medium (2× CDM) (37) supplemented with 1.5% (wt/vol) glucose. All bacteria were grown at 37°C, and if appropriate the media were supplemented with antibiotics: for *E. coli*, ampicillin (50 μg/ml) and chloramphenicol (10 μg/ml); for *L. plantarum*, chloramphenicol (10 μg/ml), erythromycin (10 or 30 μg/ml for selection or replica plating, respectively), or rifampin (50 μg/ml).

DNA manipulation techniques. Plasmid DNA was isolated from *E. coli* using Jetstar columns as recommended by the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). For DNA manipulations in *E. coli*, standard procedures were used (36). Restriction endonucleases (Invitrogen, Carlsbad, CA), T4 DNA ligase (Boehringer, Mannheim, Germany), Pfx (Promega, Leiden, The Netherlands), and KOD (Toyobo, Osaka, Japan) DNA polymerases were used as specified by the manufacturers. Primers were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Chromosomal DNA isolation, preparation of electrocompetent cells, and DNA transformation of *L. plantarum* were performed as described previously (38, 39).

Construction of NZ7102 and NZ7114. The *L. plantarum* sortase-encoding *srtA* gene (*lp_0514*) deletion was performed according to previously described methods (40). Briefly, 1.0 kb of the 5′- and 3′-flanking regions of *srtA* were amplified using chromosomal DNA of *L. plantarum* WCFS1 as the template and the primer pairs lp_0513F/lp_0513R and lp_0515F/lp_0515R, respectively. In addition, the *loxP-cat-loxP* fragment was amplified using the primers Pml-loxF and Ecl-loxR with pNZ5319 as the template. The 3 amplicons were joined by PCR using primers lp_0513F and lp_0515R, and the generated 3.0-kb DNA fusion fragment was cloned into PmeI-Ecl136II-digested pNZ5319 (40), yielding pNZ7114. This plasmid was integrated into the *L. plantarum* WCFS1 chromosome through a double-crossover event, replacing the *srtA* gene with the chloramphenicol acetyltransferase gene (P_{32}-*cat*) cassette, which was subsequently removed by the temporal expression of the Cre recombinase (40), yielding NZ7114.

For the *in vivo* survival assay, a rifampin-chloramphenicol-resistant control derivative of *L. plantarum* WCFS1-R (29) was constructed that allows selective plating after GI passage in conventional mice. The pNZ7101 (31)-derived chloramphenicol cassette was integrated in the intergenic region of the convergently oriented genes *lp_2681* and *lp_2683*. A 2.1-kb fragment of the *lp_2681-lp_2683* locus was amplified by PCR using primers HF and HR and chromosomal DNA of *L. plantarum* WCFS1 as the template, and it was cloned into pGEMt (Promega, Madison, WI), yielding pNZ7111. The P32-*cat* chloramphenicol resistance cassette of pNZ7101 was amplified by PCR using the primers P32catF and P32catR. The resulting 1.1-kb amplicon was digested with PacI and cloned into similarly digested pNZ7111, yielding pNZ7102, which was integrated into the *lp_2681-lp_2683* intergenic region of *L. plantarum* WCFS1-R (29) using a double crossover strategy (40). Colonies which displayed the correct phenotype (chloramphenicol resistance and erythromycin sensitivity) were checked for the anticipated genetic organization of the modified *lp_2681-lp_2683* locus by using the primer pairs SCO_2681 plus catR and SCO_2683 plus catF. A colony which displayed the correct phenotype and genotype was designated NZ7102.

Assessment of *in vitro* SrtA activity in *L. plantarum*. The protocol for *in vitro* assessment of SrtA activity was adapted from Ton-That et al. (2). *L. plantarum* WCFS1 and NZ7114 were grown in 2× CDM until late stationary phase. Cells were harvested by centrifugation (6,000 × *g*, 20 min, 4°C), resuspended in phosphate-buffered saline (PBS) (pH 7.5), and disrupted by bead beating (3 cycles of 30 s at 4 m/s) using a Savant FastPrep FP120 instrument (Qbiogen Inc., Illkirch, France). Samples were centrifuged (2,500 × *g*, 10 min, room temperature) to remove intact cells and beads. The supernatants were collected and centrifuged (12,000 × *g*, 10 min, 4°C), and the pelleted cell envelopes were resuspended in 50 mM Tris-HCl (pH 7.5) to an optical density at 600 nm (OD_{600}) of 2.0. The sortase substrate peptide dabcyl-GTLPQTDEQE-Edans (EZbiolab, Carmel, CA) was dissolved in Tris-HCl (pH 7.5) and added to the reaction to a final concentration of 10 μM. The strong nucleophile hydroxylamine (NH_2OH), which was previously demonstrated to enhance sortase activity *in vitro* (2), was added to a final concentration of 0.2 M. All samples were incubated at 37°C for 1 h, followed by centrifugation (12,000 × *g*, 5 min, 4°C) to remove the cell envelope material. The supernatants were analyzed in a fluorometer using excitation at 350 nm followed by assessment of emission at 495 nm.

Transcriptome analysis and data interpretation. *L. plantarum* and NZ7114 were grown in MRS and harvested at an OD_{600} of 1.0. RNA was isolated according to previously described methods (41, 42). In short, following methanol quenching (43), cells were harvested by centrifugation (6,000 × *g*, 20 min, 4°C), resuspended in 400 μl ice-cold CDM medium, and transferred to tubes containing 500 μl phenol-chloroform solution (4:1 [vol/vol]), 30 μl 10% sodium dodecyl sulfate, 30 μl 3 M sodium acetate (pH 5.2), and 0.5 g zirconium beads. Cells were disrupted by bead beating using a Savant FastPrep FP120 instrument (Qbiogen Inc., Illkirch, France), and RNA was purified from the aqueous phase using the High Pure isolation kit (Roche Diagnostics, Germany). RNA concentration and purity were determined using A_{260} and A_{280} measurements using an ND-1000 spectrometer (NanoDrop Technologies Inc., Wilmington, DE), and RNA quality was verified with a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Samples displaying a 23S/16S RNA ratio equal or superior to 1.6 were used for labeling. Three μg RNA was used for cDNA synthesis. Cyanine-3 (Cy3) and cyanine-5 (Cy5) cDNA labeling was performed as described previously (44) using the CyScribe Post-Labeling and Purification kits according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, United Kingdom). Cy dye-labeled cDNAs (0.5 μg each) were hybridized to *L. plantarum* WCFS1 printed-oligonucleotide DNA microarrays (Agilent Technologies, Amstelveen, the Netherlands). The array design and transcriptome data were deposited under platform GPL13984 and accession number GSE34999, respectively, in NCBI's Gene Expression Omnibus (GEO) (45, 46). Hybridization and scanning procedures were performed as previously described (44). Slide scanning was carried out at several photomultiplier tube (pmt) values, and the optimal scan of each individual microarray was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low-signal spots). The data were normalized using the Lowess normalization as avail-

able in MicroPrep (47). For statistical significance, the false discovery rate (FDR) was used (48) with an FDR-adjusted *P* value cutoff of 0.05 employed for genes showing at least 2-fold altered expression levels.

Surface proteome profiling of *L. plantarum*. The protocol for the surface trypsinization was adapted from Severin et al. (49). *L. plantarum* strains were grown in 2× CDM using a Sixfors fermentor system (Infors, Bottmingen, Switzerland) under constant agitation (150 rpm). The pH was maintained at 5.9 by the titration of 2.5 M NaOH. Cell cultures were harvested from fermentors (200 ml at an OD_{600} of 1.0 and 50 ml after overnight growth, which was subsequently diluted to obtain a 200-ml concentrated equivalent to an OD_{600} of 1.0) and pelleted by centrifugation (6,000 × *g*, 20 min, 4°C). Pellets were washed twice in 20 ml 20 mM Tris-HCl (pH 7.6) buffer containing 150 mM NaCl, 1 M xylose, 20 mM $CaCl_2$, and 5 mM dithiothreitol (DTT) and subsequently were resuspended in 5 ml of the same buffer, followed by the addition of 15 μg mass spectrometry-grade trypsin (T6567; Sigma-Aldrich, St. Louis, MO). Samples were incubated for 10 min at 25°C, aliquoted into Eppendorf tubes, and centrifuged (20,000 × *g*, room temperature, 1 min). The tryptic peptide-containing supernatants were collected and filtered through a cellulose-acetate filter (0.2-μm pore size, 25-mm diameter; Sigma-Aldrich). To further complete protein digestion, 5 μg of trypsin was added and the samples were incubated overnight (4 rpm, 25°C). Trypsin was inactivated by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1% (vol/vol). The samples were freeze-dried and stored at −20°C prior to mass spectrometry analysis.

Extraction of bacterial cell surface-associated proteins with LiCl. To extract noncovalently bound surface proteins, 100-ml samples of cultures of *L. plantarum* and NZ7114 grown overnight in 2× CDM were harvested by centrifugation (6,000 × *g*, 20 min, 4°C). Cell pellets were washed 3 times in PBS (pH 7.5) and subsequently incubated in 2 ml 1.5 M LiCl (20 min on ice), followed by centrifugation (12,000 × *g*, 20 min, 4°C). The supernatants were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10%, and samples were incubated for 1 h on ice followed by centrifugation (12,000 × *g*, 20 min, 4°C). The protein pellets were washed 3 times with acetone and air dried. For trypsin digestion, the protein pellet was dissolved in 6 M urea and subsequently diluted in 100 mM triethylammonium bicarbonate buffer to a final concentration of 1 M urea (final sample volume of 30 μl). For reduction and alkylation, Tris-[2-carboxyethyl]-phosphine (TCEP) was added to a final concentration of 50 mM (with incubation for 20 min at 60°C), followed by the addition of iodoacetamide (IAA) to a final concentration of 500 mM prior to incubation for 1 h at room temperature. Subsequently, 500 ng sequencing-grade trypsin (Promega, Leiden, The Netherlands) was added, and samples were incubated overnight at 37°C. Trypsin was inactivated by addition of TFA to a final concentration of 0.1% (vol/vol) prior to mass spectrometry analysis.

Mass spectrometry-based surface proteome profiling. Peptides (either derived from surface trypsinization or LiCl-based protein extraction) were separated by reverse-phase chromatography on a C_{18} capillary column (inner diameter [ID], 75 μm by 150 mm; 3-μm particle size; Dr. Maisch, Ammerbuch-Entringen, Germany) packed in house and mounted on a Proxeon Easy-LC system (Proxeon Biosystems, Odense, Denmark) in line with a trapping precolumn (ReproSil-Pur C18-AQ; ID, 100 μm by 20 mm; Proxeon). Solutions of 0.1% formic acid in water and 0.1% formic acid in 100% acetonitrile were used as mobile phases. Peptides were eluted over 140 min using a 4 to 35% acetonitrile gradient at a flow rate of 250 nl/min. Eluted peptides were analyzed using a linear ion trap-Orbitrap hybrid mass spectrometer (LTQ-Orbitrap; Thermo Fisher Scientific, Bremen, Germany). The MS raw data were submitted to Mascot (version 2.1; Matrix Science, London, United Kingdom) using the Proteome Discoverer 1.1 analysis platform (Thermo Fisher Scientific, Bremen, Germany) and searched against the *L. plantarum* proteome (3,063 entries) (32), combined with reversed entries for all protein sequences to assess false discovery rates. Peptide tolerance was set to 10 ppm and 0.8 Da for intact peptides and fragment ions, respectively. The option semitrypsin was chosen, allowing for up to 2 undigested cleavage sites. The oxidation of methionine residues and deamidation of asparagine and glutamine were specified as variable modifications. The tandem MS (MS/MS)-based peptide and protein identifications were further validated with the program Scaffold v3.0 (Proteome Software Inc., Portland, OR). Protein identifications based on at least 1 unique peptide identified by MS/MS with a confidence of identification probability higher than 95 and 80% were accepted for the surface trypsinization and LiCl samples, respectively. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (50). Significant changes in protein levels were calculated based on the spectral counts of each protein, which were normalized to the sum of all spectra detected in the individual sample.

***In vivo* gastrointestinal survival analysis.** The *in vivo* survival of NZ7102 (chloramphenicol-rifampin-resistant control strain) and the SrtA-negative derivative NZ7104 (27) were investigated in a mouse model system. Animal experiments were performed at the Institute Pasteur (Lille, France) according to guidelines 86/609/CEE of the French government. Seven-week-old female BALB/c mice were purchased from Iffa Credo (Saint-Germain-sur-l'Arbresle) and had *ad libitum* access to tap water and standard mice chow during the experiment. Overnight MRS cultures of both strains were mixed, pelleted (6,000 × *g*, 10 min, room temperature), and resuspended at 10^{10} CFU/ml. Two mice received a 100-μl dose of the bacterial mixed suspension by intragastric administration at 2 consecutive days (days −1 and 0). Individual fecal samples were collected during 5 days following the last administration (days 1 to 3 and 5) and resuspended in MRS medium followed by homogenization. Serial dilutions were plated on MRS plates containing 5 μg/ml chloramphenicol and 50 μg/ml rifampin and incubated for 72 h at 37°C. An average of 36 full-grown colonies were randomly picked per time point per mouse and were identified as NZ7102 or NZ7104 using a 3-primer colony-PCR (uniVHTP, NZ7102HTP, and NZ7104HTP; see Table S1 in the supplemental material) designed to result in a 0.5- or 0.8-kb amplicon using NZ7102 or NZ7104 cells as the template, respectively. The ratio of the NZ7102 to NZ7104 populations per time point was used as an indication for their relative *in vivo* survival and/or persistence.

Immunological cell assays. After overnight growth in 2× CDM (37), *L. plantarum* and NZ7114 were harvested by centrifugation (6,000 × *g*, 20 min, 4°C) and washed 3 times in either PBS (pH 7.5) or 1.5 M LiCl for surface protein extraction, followed by 5 washes of the cells in PBS (pH 7.5). Bacterial cells were applied to human monocyte-derived immature dendritic cells (iDCs) obtained from 3 independent buffy coats (Sanquin Blood Bank, Nijmegen, the Netherlands). The procedures of monocyte isolation and iDC differentiation were recently described in detail (44). In short, human monocytes were isolated from blood using a combination of Ficoll density centrifugation and cell separation using CD14-specific antibody-coated magnetic microbeads (Miltenyi Biotec B.V., Leiden, The Netherlands). The purity and viability of the isolated $CD14^+$ cell fraction was greater than 90 and 95%, respectively. To generate iDCs, the purified $CD14^+$ cells were cultured for 6 days in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin G (Invitrogen, Carlsbad, CA), 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), interleukin-4 (IL-4; R&D Systems, Inc., Minneapolis, MN), and granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc.). GM-CSF and IL-4 were added to differentiate the monocytes into DCs. At day 6, the iDCs (1×10^6/ml) were stimulated and *L. plantarum* strains were applied at a 1:10 iDC/bacterial cell ratio. After 48 h, supernatants were analyzed for IL-10 and IL-12p70 using a cytometric bead-based immunoassay (51) according to the manufacturer's protocol (BD Biosciences, Breda, The Netherlands). The flow-cytometric data were analyzed using the BD FCAP software.

Microarray data accession numbers. The array design and transcriptome data were deposited under platform GPL13984 and accession number GSE34999, respectively, in GEO.

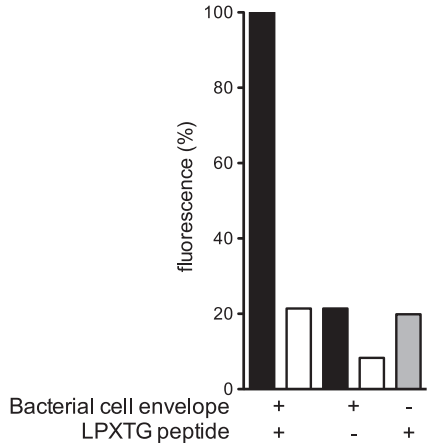

FIG 1 Dabcyl-GTLPQTDEQE-edans fluorophore peptide (LPXTG peptide) was incubated with cell envelopes of *L. plantarum* WCFS1 (black bars), its sortase-deficient derivative NZ7114 (white bars), or, as a control, without added cell envelopes (gray bar). Cleavage of the LPQTDE motif was monitored as an increase in fluorescence presented relative to the highest value obtained.

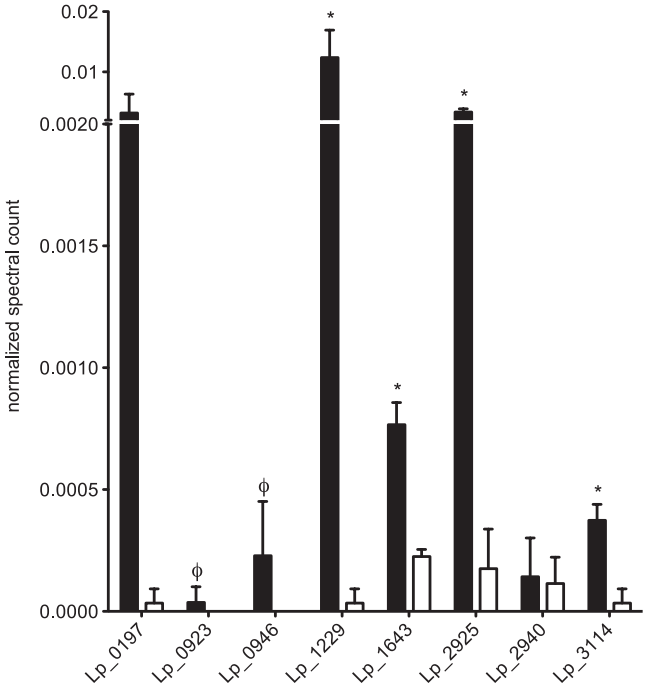

FIG 2 Normalized spectral count of proteins that were detected by surface trypsinization of intact bacterial cells of *L. plantarum* WCFS1 (black bars) and its sortase-deficient derivative NZ7114 (white bars). Data are presented as the means ± standard deviations based on 3 biological replicates (note the two-segmented *y* axis). Asterisks indicate significant differences based on a 2-tailed *t* test ($P < 0.05$). The ϕ symbol depicts proteins that were exclusively found in the wild-type surface proteome.

RESULTS

Sortase A is solely responsible for recognition of the *L. plantarum*-specific LPQTDE sorting signal, and gene disruption has only minor consequences for the bacterial transcriptome. In contrast to other lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis* IL1403 and *L. johnsonii* NCC533, that encode multiple sortase copies (34), the *L. plantarum* WCFS1 genome is predicted to encode a single sortase, i.e., *srtA* (32, 33). The encoded sortase homologue consists of 234 amino acid residues and has all the features of a typical SrtA protein, including a TLXTC motif containing the active-site cysteine residue (Cys-195) and a conserved active-site histidine (His-132) (2). To confirm this *in silico* prediction, cell envelopes of *L. plantarum* WCFS1 and its isogenic *srtA* deletion strain, NZ7114, were incubated with a chemically synthesized dabcyl-GTLPQTDEQE-edans fluorophore peptide that encompasses the conserved LPQTDE sorting signal found in most SDPs of *L. plantarum* (34, 52). Sortase activity can be monitored in this assay by an increase in fluorescence due to the sortase-dependent peptide cleavage, which separates the edans fluorophore from the dabcyl quencher (2). The addition of the fluorophore peptide to the wild-type cell envelopes led to a clear increased fluorescence signal, whereas the value obtained with the *srtA* mutant-derived envelope did not exceed background fluorescence levels as measured for samples to which either no cell envelopes or no fluorescent peptide was added (Fig. 1). This experiment confirms earlier *in silico* predictions that *srtA* encodes the sole sortase of *L. plantarum* WCFS1.

To evaluate the consequences of *srtA* deletion on the genome-wide transcriptome, we performed DNA microarray analyses which revealed that only 20 genes displayed significantly altered transcription levels in the mutant compared to the wild type (see Table S2 in the supplemental material). These include all 11 genes in the capsular polysaccharide cluster 2 (*cps2A-K*), which were upregulated (up to 62-fold) in the *srtA* deletion strain. Notably, the transcription of genes encoding SDP were not affected by the *srtA* deletion. In addition, we found that the *srtA* deletion did not influence growth rate, the final OD_{600} reached, and cell shape as investigated by light microscopy (data not shown). Taken together, our data show that the absence of sortase had only minor consequences for the *L. plantarum in vitro* physiology.

Sortase is essential for appropriate subcellular localization of SDPs. Earlier attempts to assess the impact of sortase on the subcellular localization of SDPs were typically by gel-based approaches. However, SDS-PAGE-based experiments showed that cell wall extracts for the wild type and *srtA* mutants displayed virtually identical protein band patterns (53). These data suggest that gel-based approaches are not suitable for distinguishing loosely associated and covalently coupled proteins in the cell envelope. To this end, we have confirmed with SDS-PAGE that cell envelope fractions (which include the cell wall) appeared identical for the wild type and NZ7114 (see Fig. S1 in the supplemental material). Therefore, an alternative, non-gel-based approach was employed for which wild-type and NZ7114 cells were grown until late stationary phase and exposed to surface trypsinization, followed by MS/MS-based protein identification. Using this approach, a total of 8 SDPs could be identified (Fig. 2; also see Table S3 for a complete list of SDPs). Overall, SDPs were represented by an average of 2 and 0.06% of the assigned spectra in the samples derived from the wild type and NZ7114, respectively. The subcellular localization of 2 SDPs appeared to be unaffected by the absence of sortase, as illustrated by the fact that Lp_2940 and Lp_0197 abundancy was not significantly different in the wild-type and NZ7114 surface proteome samples. In contrast, the remaining 6 detected SDPs were found to be significantly more abundant in the surface proteome fractions of the wild type than NZ7114 (Lp_1229 [Msa], Lp_1643, Lp_2925, and Lp_3114) or

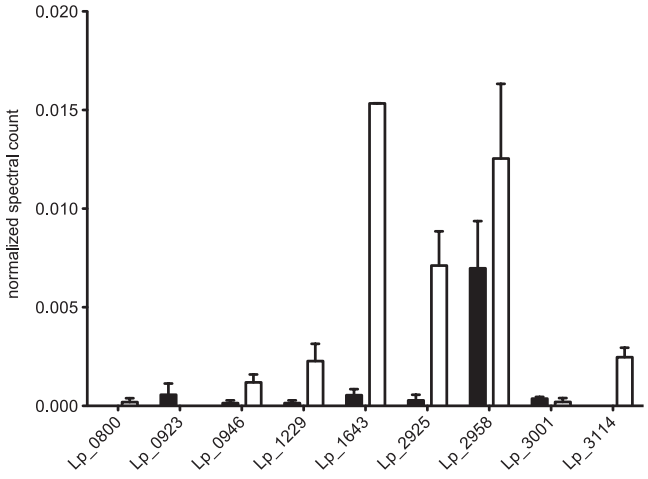

FIG 3 Normalized spectral count of proteins that were detected by LiCl surface protein extraction of intact bacterial cells of *L. plantarum* WCFS1 (black bars) and its sortase-deficient derivative NZ7114 (white bars). Data are presented as the means ± ranges between 2 biological replicates.

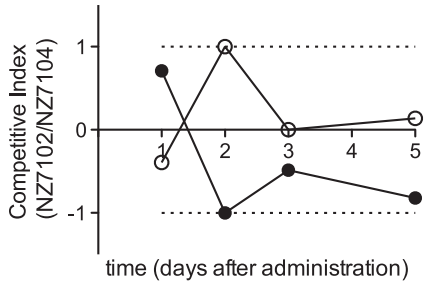

FIG 4 Competitive index (CI; on a 2-log scale) of *L. plantarum* NZ7102 and NZ7104 after GI passage in two mice (filled and open circles) assessed by antibiotic-based selective plating from fecal samples followed by genotype identification based on a discriminative 3-primer colony PCR.

were exclusively detected in the wild-type surface proteome (Lp_0923 and Lp_0946) (Fig. 2). These experiments demonstrate that although the consequences of SrtA deficiency were different for individual SDPs, sorting deficiency reduced the cell envelope abundance of the majority of the detected SDPs.

Sortase is essential for covalent coupling of SDPs to peptidoglycan. Loosely associated proteins were extracted from wild-type and NZ7114 cells by LiCl. Overall, SDPs were represented by an average of 0.9 and 4.1% of the assigned spectra in the samples derived from the wild type and NZ7114, respectively. In the LiCl-extracted fractions, 9 SDPs were identified, of which 7 were exclusively or more abundantly found in the NZ7114-derived samples (Fig. 3). These included Lp_0946, Lp_1229, Lp_1643, Lp_2925, and Lp_2958, which were detected in larger amounts, and Lp_0800 and Lp_3114, which were exclusively detected in samples derived from NZ7114. In contrast, the abundance of Lp_3001 remained unaffected by the *srtA* deletion. Lp_0923 was detected in the LiCl extraction derived from the wild type but not in that of NZ7114. These data suggest that the cell surface binding of SDPs is noncovalent in NZ7114, which generally allows SDPs to be more readily extracted by LiCl incubation than the wild-type strain.

Sortase deletion does not affect gastrointestinal survival of *L. plantarum*. The experiments described above clearly established that several SDPs are not covalently coupled to peptidoglycan in the SrtA-deficient strain NZ7104. Several of these SDPs appear to be induced in the gastrointestinal tract (see Table S3 in the supplemental material), and GI survival and persistence is considered an important trait of probiotic strains (54). We therefore determined the effect of hampered SDP sorting on this trait in a pilot experiment using two mice. The relative survival and persistence in the mouse intestinal tract was determined for *L. plantarum* NZ7102 (chloramphenicol-resistant wild type) and NZ7104 (Δ*srtA*) (27) in a competitive colonization experiment. Notably, we used the SrtA-negative chloramphenicol acetyltransferase replacement strain NZ7104 (27) in this experiment rather than NZ7114, which was used in all other experiments, as NZ7104 is chloramphenicol and rifampin resistant, allowing selective plating from fecal samples. NZ7102 and NZ7104 were administered to mice in equal amounts by gastric intubation at days −1 and 0, and the population ratio was determined on days 1 to 3 and 5 by antibiotic-based selective plating from fecal pellets, followed by colony identification by a discriminative 3-primer PCR. The PCRs on the input samples (days −1 and 0) indicated that both strains were administered in approximately equal amounts ($\log_2$ ratios were between 1 and −1; data not shown), establishing the validity of the 3-primer PCR approach while pinpointing that ratio changes larger than 2-fold up or down can be monitored with this approach. Considering these sensitivity limits, NZ7102 and NZ7104 were detected at indistinguishable levels in samples derived from fecal pellets at all time points after administration (Fig. 4). In addition, NZ7104 could readily be detected 5 days after the last administration. These observations indicate that in this relatively small experiment, SrtA deficiency and the associated altered subcellular localization for SDPs did not affect GI survival and/or persistence of *L. plantarum*.

The presence of SDPs affects immune responses in human immature dendritic cells. Modulation of immune responses has been proposed as one of the prominent actions by which probiotics can beneficially affect their hosts (55, 56). To investigate the role of SDPs in immunomodulation, human monocyte-derived iDCs were cocultured with *L. plantarum* WCFS1 or NZ7114 cells that had been washed with PBS (control) or 1.5 M LiCl (removal of noncovalently bound proteins). When washed with PBS, both strains elicited similar amounts of IL-10 and IL-12p70 from iDCs (Fig. 5). Similarly, IL-10 levels detected when employing LiCl-washed WCFS1 or NZ7114 cells were similar to those of the cells treated with PBS. However, while IL-12p70 levels induced by LiCl-treated and control-treated wild-type cells were similar, IL-12p70 levels significantly increased (12-fold) after coculture with NZ7114 (Fig. 5). These data suggest a role of SDPs in attenuation of proinflammatory responses, either directly or indirectly by shielding the exposure of proinflammatory compounds present in the cell envelope, such as teichoic acids and/or peptidoglycan.

DISCUSSION

Sortases mediate the covalent anchoring of proteins to the cell wall peptidoglycan of Gram-positive bacteria (1) and have been shown to facilitate the interactions between bacteria and their environments. As could be anticipated from earlier *in silico* observations (34), deletion of *srtA* in *L. plantarum* WCFS1 resulted in complete abolishment of sortase activity, while it elicited only minor transcriptome consequences under laboratory conditions. Impor-

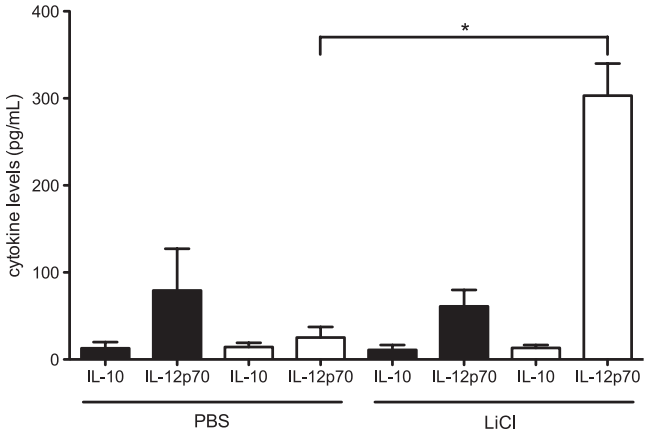

FIG 5 IL-10 and IL-12p70 secretion pattern by monocyte-derived immature dendritic cells after stimulation with *L. plantarum* WCFS1 (black bars) or its sortase-deficient derivative NZ7114 (white bars). Data are presented as the means ± standard deviations based on 3 donors. An asterisk indicates significant differences based on a 2-tailed *t* test ($P < 0.05$).

tantly, although 6 (out of 8) detected SDPs could be identified by surface trypsinization in both the parent and *srtA* mutant strain, *srtA* deletion led to a significant decrease in the relative abundance of the SDPs. This observation indicates that while SDPs may not be coupled covalently to the peptidoglycan in the absence of sortase activity, they remain surface localized, possibly by stop-transfer-mediated anchoring in the cytoplasmic membrane (53). However, as shown by LiCl extraction, the majority of SDPs were loosely associated with the cell surface, suggesting that the hydrophobic C-terminal stop-transfer domain does not allow robust membrane anchoring, which consequently results in the release of the SDPs.

In *L. plantarum* WCFS1, the absence of SrtA did not affect intestinal survival and/or persistence in mice. It should be noted that it remains to be established whether our work using a murine model can be extrapolated to the human situation. Considering that only two animals were used in this pilot study, it appears difficult to draw solid conclusions from this experiment. However, it should be noted that our experiment assesses gastrointestinal survival/persistence in a competitive experiment (animals received both the wild-type and sortase mutant strains concomitantly), which increases the significance of these observations. The array data on the clean sortase mutant used in the other experiments (NZ7114) suggest that expression of the surrounding genes (*lp_0513* and *lp_0515*) is unaltered compared to the wild type (see Table S2 in the supplemental material). Despite these observations, we cannot rule out polar effects in the chloramphenicol replacement mutant (NZ7104) used for the gastrointestinal survival assay for its convenient antibiotic-based selection from feces. Nevertheless, our results corroborate earlier results obtained for *L. johnsonii* NCC533 and its sortase-deficient derivative (22). At first glance, our result appears to contradict the observation that an *L. plantarum* WCFS1 mutant that lacked the SDP Lp_2940 displayed lower survival/persistence rates in the mouse GI tract (31). However, our surface proteome analyses revealed that *srtA* mutation did not affect surface exposure of Lp_2940, which could explain the unaffected intestinal survival phenotype in strain NZ7104. In addition, Lp_2940 was not extracted by LiCl from cells of the *srtA* deletion mutant, which suggests that this protein is still anchored within the cell envelope. Contrary to this, previous work has shown that deletion of either *msa* or *srtA* resulted in a complete loss of *L. plantarum* mannose-specific adherence (27). Analogously, our surface trypsinization approach revealed that the surface exposure of Msa is severely decreased in the SrtA-deficient strain, which could explain the impact of the *srtA* gene deletion on mannose-specific adhesion capacity (27). These results clearly show that the consequences of SrtA deficiency may vary for different SDPs. Such variations may depend on folding and/or stability characteristics of the SDP protein when its biogenesis is impaired by the lack of sortase activity or could relate to differences in the capacity of the C-terminal stop-transfer sequences of the different SDPs to anchor them robustly in the cytoplasmic membrane and thereby sustain their surface exposure and function.

Coculturing of human monocyte-derived iDCs with *L. plantarum* WCFS1 or its SrtA-deficient derivative revealed that LiCl extraction of bacterial cells strongly affected the proinflammatory signaling properties of the SrtA-deficient strain while the wild type remained unaffected. More specifically, the LiCl-treated cells of the *srtA* mutant strain strongly stimulated secretion of the proinflammatory cytokine IL-12p70. This may be directly caused by the lower abundance of one or more SDPs in these cells or may be due to a loss of shielding of secondary cell-signaling molecules by SDP proteins (57). The observation that the abundance of SDPs on the cell surface of *srtA* mutant cells is generally much lower than their levels encountered in wild-type cells suggest that these proteins have an active role in immunomodulation rather than through shielding of other cell envelope components. However, the induction of the expression of the *cps2* gene cluster in the *srtA* mutant may lead to a higher level of cell surface glycan production, which in its turn might contribute to shielding of immunomodulatory cell envelope components and/or altered levels of phagocytosis due to changes in surface properties. In conclusion, this work demonstrates the importance of assessing the full repertoire of potential host (immune) cell signaling components of the bacterial cell envelope, which include the sortase-dependent surface proteome and the more extensively studied cell envelope polymers, i.e., peptidoglycan (58, 59) and lipoteichoic acid (60–62).

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