



Identification of Genes Required for Glucan Exopolysaccharide Production in *Lactobacillus johnsonii* Suggests a Novel Biosynthesis Mechanism

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ABSTRACT *Lactobacillus johnsonii* F19785 makes two capsular exopolysaccharides—a heteropolysaccharide (EPS2) encoded by the *eps* operon and a branched glucan homopolysaccharide (EPS1). The homopolysaccharide is synthesized in the absence of sucrose, and there are no typical glucansucrase genes in the genome. Quantitative proteomics was used to compare the wild type to a mutant where EPS production was reduced to attempt to identify proteins associated with EPS1 biosynthesis. A putative bactoprenol glycosyltransferase, F19785_242 (242), was less abundant in the Δ *eps*_{cluster} mutant strain than in the wild type. Nuclear magnetic resonance (NMR) analysis of isolated EPS showed that deletion of the F19785_242 gene (242) prevented the accumulation of EPS1, without affecting EPS2 synthesis, while plasmid complementation restored EPS1 production. The deletion of 242 also produced a slow-growth phenotype, which could be rescued by complementation. 242 shows amino acid homology to bactoprenol glycosyltransferase GtrB, involved in O-antigen glycosylation, while *in silico* analysis of the neighboring gene 241 suggested that it encodes a putative flippase with homology to the GtrA superfamily. Deletion of 241 also prevented production of EPS1 and again caused a slow-growth phenotype, while plasmid complementation reinstated EPS1 synthesis. Both genes are highly conserved in *L. johnsonii* strains isolated from different environments. These results suggest that there may be a novel mechanism for homopolysaccharide synthesis in the Gram-positive *L. johnsonii*.

IMPORTANCE Exopolysaccharides are key components of the surfaces of their bacterial producers, contributing to protection, microbial and host interactions, and even virulence. They also have significant applications in industry, and understanding their biosynthetic mechanisms may allow improved production of novel and valuable polymers. Four categories of bacterial exopolysaccharide biosynthesis have been described in detail, but novel enzymes and glycosylation mechanisms are still being described. Our findings that a putative bactoprenol glycosyltransferase and flippase are essential to homopolysaccharide biosynthesis in *Lactobacillus johnsonii* F19785 indicate that there may be an alternative mechanism of glucan biosynthesis to the glucansucrase pathway. Disturbance of this synthesis leads to a slow-growth phenotype. Further elucidation of this biosynthesis may give insight into exopolysaccharide production and its impact on the bacterial cell.

KEYWORDS exopolysaccharide, alpha glucan, *Lactobacillus johnsonii*, proteomics, glycosyltransferase, nuclear magnetic resonance

Production of exopolysaccharides (EPS) has a large impact on the nature of the bacterial surface and hence on interactions with the environment, hosts and host defense systems, and other microbes (1, 2). EPS can protect bacteria against environ-

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mental conditions, both outside and inside the host (1, 3, 4), and in the case of pathogens such as *Streptococcus pneumoniae*, they can have an important association with immune evasion and virulence (5). EPS can have immunomodulatory and protective properties in the host (6–9) and can affect the composition and function of the gut microbiota (10, 11). EPS can also play a crucial role in biofilm formation, adhesion to host cells, and colonization (3, 12–15). In addition to their biological importance, bacterial EPS have a range of technological applications in food, pharmaceutical, and other industries and may also have potential health benefits due to their activities in immune stimulation, antitumor activity, and lowering of blood cholesterol or as prebiotics (1, 2, 16, 17).

Lactobacillus johnsonii FI9785 is a poultry isolate which has shown promise as a competitive exclusion agent against *Clostridium perfringens* (18) and *Campylobacter jejuni* (19). This strain makes 2 capsular exopolysaccharides—EPS2, a heteropolysaccharide containing glucose and galactose encoded by a 14-gene *eps* operon of the Wzx/Wzy type, and EPS1, a branched dextran homopolysaccharide with an α -(1→6) backbone and α -(1→2) branches which are present on every unit of the backbone and consist of a single glucose (Glc) residue (20, 21). This is an unusual structure which has not been described in other bacteria, although a small percentage of α -(1→2) branches were seen in dextran produced by *Leuconostoc citreum* E497 (22). Glucansucrases have been shown to synthesize homopolysaccharides in lactic acid bacteria, using sucrose as a substrate (17). However, *L. johnsonii* FI9785 makes EPS1 in the absence of sucrose, and there is no glucansucrase gene present in the genome, suggesting a different mode of biosynthesis (20). In previous work, the 14-gene *eps* operon (loci FI9785_1170 to FI9785_1183 inclusive, now renamed FI9785_RS05260 to FI9785_RS05325) was removed by deletion mutagenesis to create the mutant strain Δ *eps_cluster* (20), and a second mutant strain where just the transcriptional regulator *epsA* (FI9785_1183) was deleted was also constructed (23). Although these mutations were expected to just affect the synthesis of EPS2 and not EPS1, these strains did not show an EPS layer by transmission electron microscopy (TEM), and nuclear magnetic resonance (NMR) analysis of EPS extractions failed to identify either EPS1 or EPS2 (20, 23, 24). In this work, we compared the proteome of the wild-type *L. johnsonii* FI9785 EPS producer with the Δ *eps_cluster* mutant strain to attempt to identify proteins involved in homopolysaccharide biosynthesis.

RESULTS

Comparative quantitative proteomic analyses identified proteins affected by deletion of the *eps* cluster. In order to identify proteins involved in EPS biosynthesis, the proteome of the wild type was compared to that of a mutant with a reduced EPS capsule to highlight proteins which were missing or downregulated in the mutant. Proteomic analysis of the soluble fractions of *L. johnsonii* FI9785 and the Δ *eps_cluster* strain identified several proteins which were differently expressed between the two strains. The protein samples were trypsin digested and labeled by iTRAQ (isobaric tag for relative and absolute quantitation) reagents, mixed and analyzed using nano-liquid chromatography tandem mass spectroscopy (nLC MS/MS) or directly analyzed without labeling for the label-free experiment. Andromeda analyses resulted in the identification of 699 soluble proteins (see Data Set S1 in the supplemental material), 49 of which were differentially expressed in the Δ *eps_cluster* strain versus the wild type (WT; Table 1). The volcano plots in Fig. 1 show the proteins which changed in abundance, obtained in iTRAQ (Fig. 1A) and label-free (Fig. 1B) experiments. The two different quantitative approaches allowed the quantitation of identical proteins with a similar ratio in the mutant and the control, e.g., D0R1R2, supporting the accuracy of the analyses, but also identified different proteins, allowing an in-depth characterization of proteins altered in the Δ *eps_cluster* strain. A total of 20 proteins were found at a higher level in the Δ *eps_cluster* strain, 4 identified by iTRAQ and 17 by the label-free approach, with only one found by both methods; the remaining 29 proteins were at higher levels in the WT, 17 found by iTRAQ and 14 by the label-free method, with 2 proteins identified by both

TABLE 1 Quantified *Lactobacillus johnsonii* proteins using MaxQuant software in iTRAQ and label-free experiments^a

| Protein accession no. | Protein name | Gene name | No. of razor and unique peptides | Mol wt (kDa) | Score | log ₂ (D/WT) | D/WT ratio | WT/D ratio | iTRAQ ^b | Label free ^b | GO biological process |
|-----------------------|---|--------------------|----------------------------------|--------------|-------|-------------------------|------------|------------|--------------------|-------------------------|--|
| D0R1P2 | Uncharacterized protein | <i>F19785_401</i> | 4 | 22 | 234 | 3.71 | 13.12 | 0.08 | X | | Cell redox homeostasis; oxidation/reduction process; |
| D0R498 | Thiol peroxidase | <i>tpx</i> | 3 | 18 | 29 | 2.41 | 5.30 | 0.19 | | X | cellular oxidant detoxification |
| D0R5C5 | Ribosomal silencing factor RsfS | <i>rsfS</i> | 3 | 14 | 111 | 2.22 | 4.67 | 0.21 | X | | Mature ribosome assembly; negative regulation of ribosome biogenesis; negative regulation of translation; regulation of translation |
| D0R3E4 | 50S ribosomal protein L28 | <i>rplB</i> | 3 | 7 | 26 | 2.17 | 4.49 | 0.22 | X | | Translation |
| D0R3T4 | Aspartate-tRNA ligase | <i>aspS</i> | 26 | 71 | 155 | 1.38 | 2.60 | 0.39 | | X | Translation; tRNA aminoacylation for protein translation; aspartyl-tRNA aminoacylation |
| D0R3V1 | Glycine-tRNA ligase beta subunit | <i>glyS</i> | 16 | 78 | 150 | 1.23 | 2.35 | 0.43 | | X | Translation; arginyl tRNA aminoacylation; glycyl tRNA aminoacylation |
| D0R277 | Uncharacterized protein | <i>F19785_219</i> | 7 | 17 | 87 | 1.16 | 2.24 | 0.45 | | X | — |
| D0R5K0 | Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit C | <i>gatC</i> | 3 | 12 | 42 | 1.12 | 2.17 | 0.46 | | X | Translation; regulation of translational fidelity |
| D0R6B7 | Deoxynucleoside kinase | <i>dgk1</i> | 10 | 25 | 60 | 1.05 | 2.07 | 0.48 | | X | Nucleobase-containing compound metabolic process; deoxyribonucleoside monophosphate biosynthetic process; nucleotide biosynthetic process; phosphorylation |
| D0R362 | Ribokinase | <i>rbsK</i> | 16 | 33 | 146 | 1.04 | 2.05 | 0.49 | | X | Carbohydrate metabolic process; D-ribose metabolic process; phosphorylation; D-ribose catabolic process; carbohydrate phosphorylation |
| D0R1J3 | 30S ribosomal protein S12 | <i>rpsL</i> | 9 | 15 | 134 | 0.93 | 1.90 | 0.53 | X | | Translation |
| D0R2C4 | Recombination protein RecR | <i>recR</i> | 2 | 22 | 44 | 0.89 | 1.85 | 0.54 | | X | DNA repair; DNA recombination; cellular response to DNA damage stimulus |
| D0R4S3 | Isoleucine-tRNA ligase | <i>ileS</i> | 16 | 107 | 100 | 0.81 | 1.75 | 0.57 | | X | Translation; tRNA aminoacylation for protein translation; isoleucyl tRNA aminoacylation; aminoacyl-tRNA metabolism involved in translational fidelity |
| D0R1L4 | 30S ribosomal protein S5 | <i>rpsE</i> | 15 | 19 | 278 | 0.77 | 1.71 | 0.59 | | X | Translation |
| D0R5T0 | Uncharacterized protein | <i>F19785_1588</i> | 10 | 21 | 53 | 0.76 | 1.70 | 0.59 | | X | — |
| D0R434 | Asparagine-tRNA ligase | <i>asnS</i> | 18 | 50 | 72 | 0.74 | 1.67 | 0.60 | | X | Translation; tRNA aminoacylation for protein translation; asparagyl tRNA aminoacylation |
| D0R3G2 | 30S ribosomal protein S16 | <i>rpsP</i> | 5 | 11 | 227 | 0.73 | 1.66 | 0.60 | | X | Translation |
| D0R5D2 | 50S ribosomal protein L35 | <i>rplM</i> | 7 | 8 | 80 | 0.69 | 1.61 | 0.62 | | X | Translation |
| D0R4W9 | ATP synthase subunit b | <i>atpF</i> | 7 | 18 | 42 | 0.65 | 1.57 | 0.64 | | X | ATP biosynthetic process; ion transport; ATP synthesis coupled proton transport; ATP hydrolysis coupled cation transmembrane transport |
| D0R5D1 | 50S ribosomal protein L20 | <i>rplT</i> | 8 | 13 | 101 | 0.63 | 1.55 | 0.65 | | X | Ribosomal large subunit assembly; translation |
| D0R608 | Chromosome partitioning protein ParB | <i>parB</i> | 6 | 33 | 51 | −3.26 | 0.10 | 9.60 | X | | Pseudouridine synthesis; RNA modification |
| D0R4H4 | Pseudouridine synthase | <i>F19785_1123</i> | 5 | 27 | 115 | −3.02 | 0.12 | 8.09 | X | | Translation; translational elongation; peptide biosynthetic process |
| D0R5U7 | Elongation factor P | <i>efp</i> | 8 | 21 | 105 | −2.99 | 0.13 | 7.96 | X | | — |
| D0R1R2 | Putative glycosyl transferase | <i>F19785_242</i> | 8 | 35 | 46 | −2.50 | 0.18 | 5.64 | X | | — |

(Continued on following page)

TABLE 1 (Continued)

| Protein accession no. | Protein name | Gene name | No. of razor and unique peptides | Mol wt (kDa) | Score | log ₂ (D/WT) | D/WT ratio | WT/D ratio | iTRAQ ^b | Label free ^b | GO biological process |
|-----------------------|---|--------------------|----------------------------------|--------------|-------|-------------------------|------------|------------|--------------------|-------------------------|---|
| D0R254 | Extracellular solute-binding protein PhnD | <i>phnD</i> | 4 | 34 | 86 | -2.26 | 0.21 | 4.78 | X | | Transmembrane transport |
| D0R5M6 | Aggregation promoting factor | <i>apf2</i> | 3 | 33 | 190 | -2.04 | 0.24 | 4.12 | | X | — |
| D0R1L2 | 50S ribosomal protein L6 | <i>rplF</i> | 12 | 19 | 205 | -2.01 | 0.25 | 4.04 | X | | Translation |
| D0R588 | Peptide chain release factor 3 | <i>prfC</i> | 10 | 59 | 63 | -1.99 | 0.25 | 3.97 | X | | Translation; translational termination; regulation of translational termination |
| D0R5Z4 | Tagatose-6-phosphate kinase | <i>fruB</i> | 4 | 33 | 153 | -1.96 | 0.26 | 3.88 | X | | Carbohydrate metabolic process; lactose metabolic process; phosphorylation; carbohydrate phosphorylation |
| D0R4W2 | MreB-like protein | <i>mbi</i> | 8 | 35 | 318 | -1.83 | 0.28 | 3.56 | X | | Cell morphogenesis |
| D0R4I9 | Ribonuclease Z | <i>rnZ</i> | 4 | 35 | 17 | -1.82 | 0.28 | 3.52 | X | | tRNA processing; tRNA 3'-trailer cleavage, endonucleolytic; tRNA 3'-trailer cleavage; RNA phosphodiester bond hydrolysis, endonucleolytic |
| D0R1L5 | 50S ribosomal protein L30 | <i>rpmD</i> | 2 | 6 | 37 | -1.81 | 0.29 | 3.50 | X | X | Translation |
| D0R1U3 | Tryptophan-tRNA ligase | <i>trpS</i> | 8 | 39 | 269 | -1.80 | 0.29 | 3.47 | X | | Translation; tRNA aminoacylation for protein translation; tryptophanyl tRNA aminoacylation |
| D0R5K3 | ATP-dependent DNA helicase | <i>parA</i> | 18 | 84 | 294 | -1.60 | 0.33 | 3.04 | X | | DNA unwinding involved in DNA replication |
| D0R2Q2 | 30S ribosomal protein S6 | <i>rpsF</i> | 9 | 12 | 255 | -1.60 | 0.33 | 3.03 | X | | Translation |
| D0R268 | Putative secreted protein | <i>Fl9785_210</i> | 21 | 102 | 323 | -1.55 | 0.34 | 2.93 | | X | — |
| D0R3I4 | Proline-tRNA ligase | <i>proS</i> | 16 | 63 | 297 | -1.38 | 0.39 | 2.60 | | X | Translation; tRNA aminoacylation for protein translation; prolyl tRNA aminoacylation; aminoacyl-tRNA metabolism involved in translational fidelity |
| D0R4U6 | Valine-tRNA ligase | <i>valS</i> | 20 | 101 | 323 | -1.37 | 0.39 | 2.59 | X | | Translation; tRNA aminoacylation for protein translation; valyl tRNA aminoacylation; aminoacyl-tRNA metabolism involved in translational fidelity |
| D0R662 | Phosphoenolpyruvate-dependent sugar phosphotransferase system EIAB, probably mannose specific | <i>manL</i> | 13 | 36 | 323 | -1.16 | 0.45 | 2.24 | | X | Phosphoenolpyruvate-dependent sugar phosphotransferase system; carbohydrate transmembrane transport |
| D0R1P7 | Muramidase | <i>Fl9785_225</i> | 8 | 64 | 323 | -1.03 | 0.49 | 2.04 | | X | Metabolic process; peptidoglycan catabolic process; cell wall macromolecule catabolic process |
| D0R3J0 | Translation initiation factor IF-2 | <i>infB</i> | 13 | 99 | 122 | -1.02 | 0.49 | 2.03 | | X | Translation; translational initiation |
| D0R383 | Methionine aminopeptidase | <i>pepM</i> | 10 | 30 | 323 | -0.96 | 0.52 | 1.94 | X | | Proteolysis; protein initiator methionine removal |
| D0R501 | Probable transcriptional regulatory protein Fl9785_1304 | <i>Fl9785_1304</i> | 5 | 27 | 99 | -0.88 | 0.54 | 1.84 | | X | Regulation of transcription, DNA-templated |
| D0R4I0 | Pyruvate kinase | <i>pyk</i> | 35 | 64 | 323 | -0.88 | 0.54 | 1.84 | | X | Glycolytic process; phosphorylation |
| D0R395 | Amino-peptidase | <i>pepN</i> | 13 | 96 | 140 | -0.87 | 0.55 | 1.83 | | X | Proteolysis |
| D0R2B3 | 50S ribosomal protein L10 | <i>rplJ</i> | 9 | 21 | 191 | -0.85 | 0.56 | 1.80 | | X | Translation; ribosome biogenesis |
| D0R3F4 | Oligopeptide-binding protein OppA | <i>oppA</i> | 7 | 65 | 50 | -0.81 | 0.57 | 1.76 | X | | Transmembrane transport |
| D0R1L8 | Adenylate kinase | <i>adk</i> | 13 | 24 | 323 | -0.74 | 0.60 | 1.67 | | X | Nucleoside-containing compound metabolic process; nucleotide biosynthetic process; phosphorylation; AMP salvage; nucleoside monophosphate phosphorylation |
| D0R5L2 | NH(3)-dependent NAD(+) synthetase | <i>nadE</i> | 6 | 31 | 149 | -0.71 | 0.61 | 1.63 | | X | NAD biosynthetic process |

^aWT, *L. johnsonii* Fl9785; D, *Δeps* cluster; GO, gene ontology; —, no process identified.
^bX, protein identified as having significantly different abundances between D and WT with this treatment.

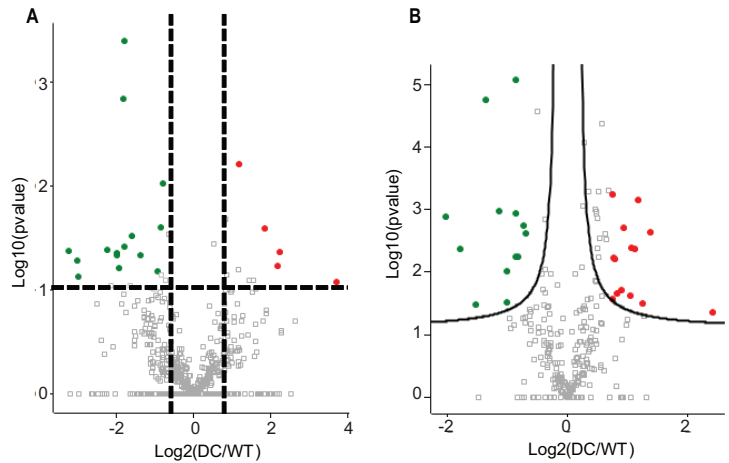


FIG 1 Volcano plots of differentially expressed proteins. Results compare *L. johnsonii* Δ *eps_cluster* (DC) versus F19785 (WT) obtained using a two-sided *t* test in panels A (iTRAQ) and B (label-free experiments). Red indicates abundance higher in DC than WT; green indicates abundance lower in DC than WT (using a *P* value of less than 0.05).

methods (Table 1). In Fig. 2, enriched Gene Ontology (GO) terms of proteins found at different levels in the *L. johnsonii* F19785 and Δ *eps_cluster* strains are described. Soluble proteins, mainly present in the cytoplasm, are involved in ATP binding (GO:0005524), translation (GO:0006412), nucleotide binding (GO:0000166), and transferase activity (GO:0016740) in the mutant strain. Almost half of the proteins with altered abundance were associated with ribosomal structure, translation, and protein biosynthesis, but

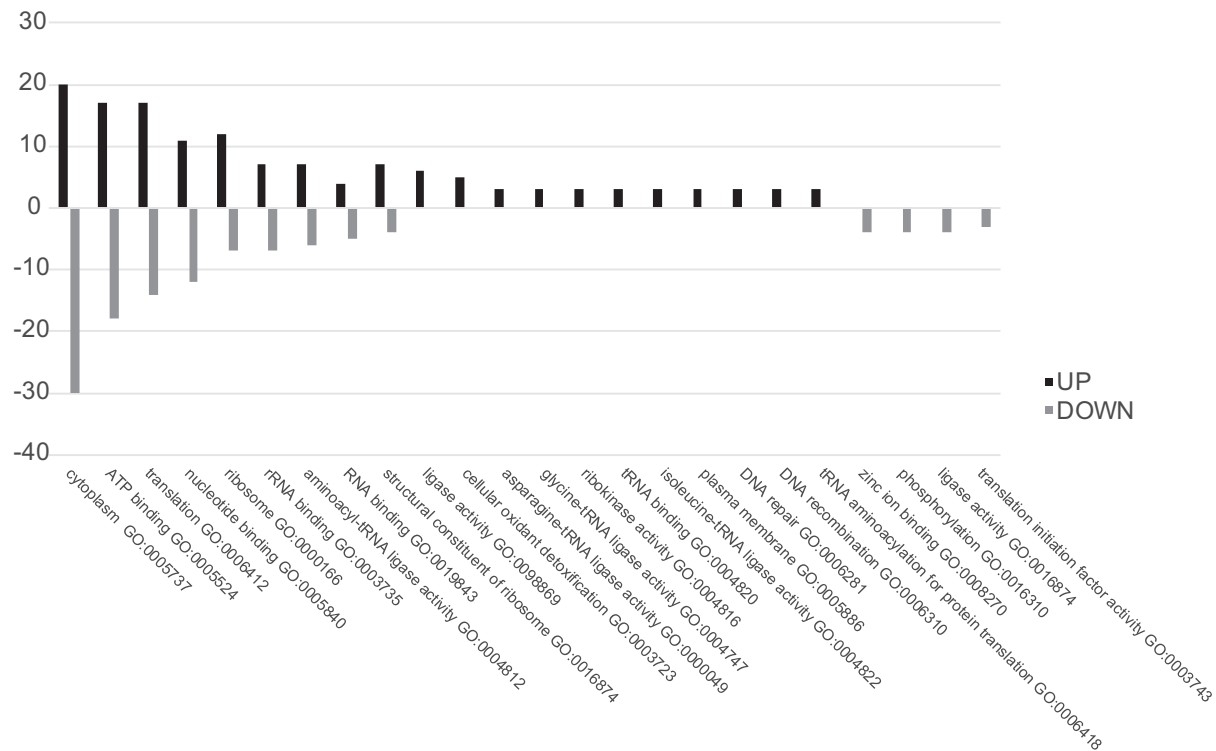


FIG 2 Gene Ontology analyses of differentially expressed proteins. On the x axis, the Gene Ontology enriched terms are shown, and on the y axis, the percentage of enrichment is shown. Up, processes enriched in the Δ *eps_cluster* mutant strain compared to the WT; Down, processes enriched in the WT compared to the mutant.

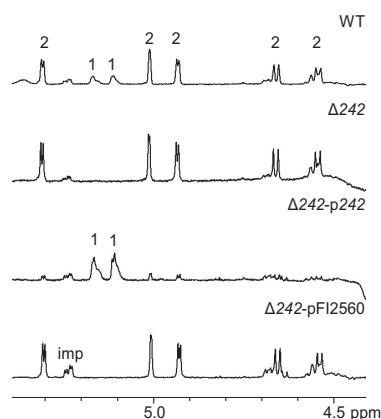


FIG 3 NMR analysis of pellet-associated EPS. 600 MHz ^1H NMR spectra of EPS from WT and modified *L. johnsonii* (pellet samples, D_2O , 338°K). Anomeric signals of EPS1 and EPS2 are labeled 1 and 2, respectively; imp, impurities. The presence of EPS1 is indicated by two H1 signals of equal intensity at 5.17 ppm [(1,2,6)- α -Glc] and 5.11 ppm (t- α -Glc). There are multiple H1 signals associated with EPS2 as indicated at the chemical shifts listed previously (20, 23).

some were more and some less abundant in the $\Delta\text{eps_cluster}$ strain, with no discernible pattern. No other biological processes seemed to be strongly impacted in the $\Delta\text{eps_cluster}$ strain. Although EPS is known to protect the cells from stress, there were no notable changes in stress response except a higher level of thiol peroxidase, commonly involved in cell redox homeostasis (Table 1).

One protein found at a lower level in the $\Delta\text{eps_cluster}$ strain—D0R1R2, encoded by *FI9785_242* (242; renamed *FI9785_RS00855*)—was identified using Rapid Annotations using Subsystems Technology (RAST) analysis as a bactoprenol glycosyltransferase, which is involved in cell wall biosynthesis. This was one of the three proteins identified by both iTRAQ and the label-free protocol. Blastp analysis indicated homology to the glycosyltransferase 2 superfamily, particularly to domains cd04187 (DPM1-like bac; expected value [E-value], $7.24\text{e}-81$), the PRK10714 superfamily (undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase; E-value, $1.28\text{e}-33$), pfam00535 (glycosyltransferase family 2; E-value, $6.63\text{e}-28$), and COG0463 (glycosyltransferase involved in cell wall biosynthesis; E-value, $2.2\text{e}-26$). This protein was selected for gene deletion to investigate a possible role in EPS1 biosynthesis.

Deletion of 242 prevents biosynthesis of homopolysaccharide EPS1. The coding sequence for *FI9785_242* (242) was deleted from the *L. johnsonii* *FI9785* genome to create strain $\Delta 242$. Comparison of proton nuclear magnetic resonance (^1H -NMR) profiles of EPS extracted from the WT and strain $\Delta 242$ showed that EPS1 production was undetectable in samples extracted both from cell pellets and from supernatants (Fig. 3; Fig. S1A), indicating that 242 is essential for EPS1 production. NMR analysis of EPS extracted from a derivative of strain $\Delta 242$ containing a plasmid expressing the 242 gene under the regulation of a strong constitutive promoter ($\Delta 242\text{-p}242$) showed that complementation restored EPS1 expression, with an increased ratio of EPS1 to EPS2 compared to the WT (Fig. 3; Fig. S1A).

Previous NMR analysis of EPS extracted from the $\Delta\text{eps_cluster}$ and ΔepsA strains and then purified by trichloroacetic acid (TCA) precipitation failed to detect EPS1 or EPS2 (20, 23). However, our analysis here of crude EPS preparations prior to TCA purification, using an increased temperature and higher number of scans, revealed the presence of EPS1 in both strains (Fig. S1B). This indicates that the genes in the *eps* cluster which produce EPS2 are not required for EPS1 production.

241-242 show homology to GtrA-GtrB and have homologues in Gram-positive bacteria. Blastp analysis showed that amino acid homologues of 242 are widely distributed among *Lactobacillus* spp., with a high conservation of amino acid sequence (71 to 100% in the first 70 matches). Alignment of 242 with GtrB proteins from *Shigella*

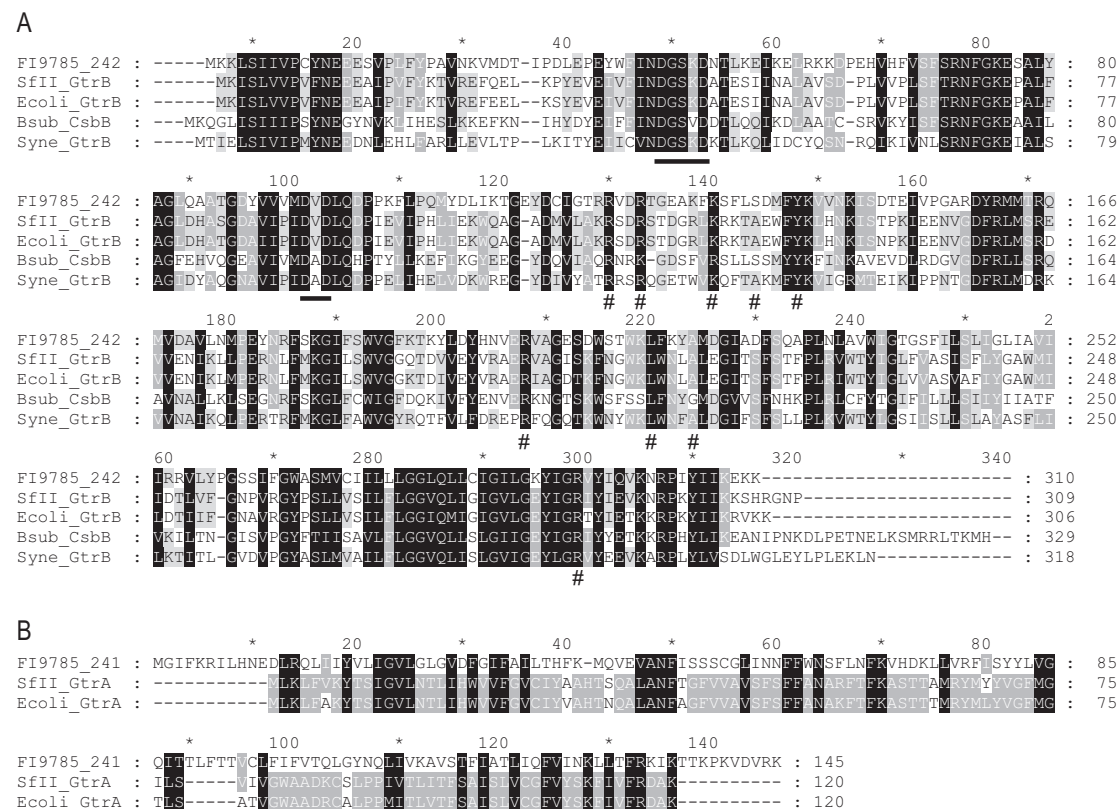


FIG 4 Amino acid alignments with GtrA and GtrB proteins. (A) Translation of 242 coding sequence (NCBI reference sequence [WP_012845545](#)) aligned with GtrB proteins from *Shigella* phage SfII (NCBI Protein accession number [YP_008318506](#) [52]), *E. coli* K-12 (NCBI Protein accession number [P77293](#) [53]), *B. subtilis* CsbB (NCBI Protein accession number [Q45539](#) [54]) and *Synechocystis* sp. strains (NCBI Protein accession number [Q55487](#) and Protein Data Bank number [5EKP](#) [25]). Conserved motifs DXD and DXSXD are underlined, and residues affecting activity in 5EPK are marked with a #. (B) Translation of the 241 coding sequence (NCBI reference sequence [WP_004896037](#)) aligned with GtrA family proteins from *Shigella* phage SfII (NCBI Protein accession number [YP_008318507](#) [52]) and *E. coli* K-12 (NCBI Protein accession number [P77682](#) [53]). Black, dark gray, and light gray indicate 100%, 80%, and 60% homology, respectively.

phage SfII and *Escherichia coli*, a putative bactoprenol glycosyltransferase CsbB from *Bacillus subtilis*, and a polyisoprenyl-phosphate glycosyltransferase from a *Synechocystis* sp. whose crystal structure has been solved (25) shows areas of homology across the whole sequence, including the motifs DXD and DXSXD, which have previously been identified as being conserved in glycosyltransferases (25–27) (Fig. 4A). Mutation of selected amino acids in the *Synechocystis* sp. GtrB was previously shown to affect enzymatic activity (25); all but one of these amino acids are conserved in 242 (Fig. 4A).

Blastp analysis of the translated product of the gene upstream of 242, *FI9785_241* (241; renamed *FI9785_RS00850*), shows homology to domains pfam04138 (GtrA-like protein; E-value, $3.04e-18$) and COG2246 (putative flippase GtrA; E-value, $2.78e-07$). When aligned to the GtrA sequence pairing the SfII and *E. coli* GtrBs, 241 shows some conservation of sequence but less than that seen with the GtrB counterparts (Fig. 4B). GtrAB pairs have been identified in a range of Gram-negative bacteria and their bacteriophages and are commonly found with a glycosyltransferase GtrX, with the three-protein complex engineering the glycosylation of O-antigens with a single sugar moiety (28). However, we could not identify any further glycosyltransferases in the *L. johnsonii* FI9785 genome in the immediate vicinity of 241 and 242.

The 241–242 pair and surrounding genes show strong nucleotide conservation in other strains of *L. johnsonii* isolated from different sources. A surrounding 11.1-kb section encompassing 15 open reading frames (ORFs) from *L. johnsonii* FI9785 was compared with equivalent regions from annotated genomes of strains isolated from the human gut (NCC533), pig intestine (DPC6026), rat feces (N6.2), turkey (UMNLJ22),

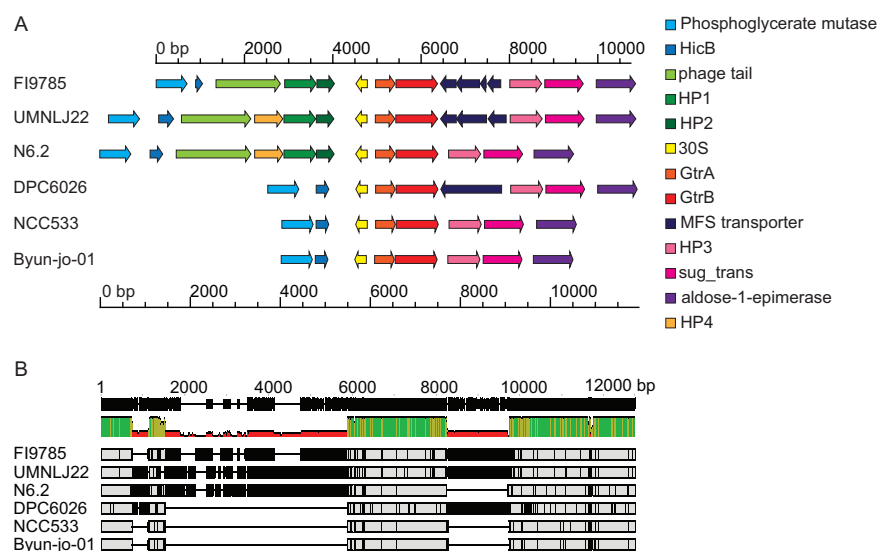


FIG 5 Conservation of genes with *L. johnsonii* strains from different environments. (A) ORFs are shown from genomes of *L. johnsonii* strains FI9785 (GenBank accession number [FN298497](#) [49], nucleotides 184194 to 194938, loci *FI9785_RS00820* to *FI9785_RS00875*), UMN LJ22 (GenBank accession number [NZ_CP021704](#) [T. J. Johnson and B. Youmans, unpublished], nucleotides 699996 to 711750, loci *A3P32_RS03290* to *A3P32_RS03350*); N6.2 (GenBank accession number [NC_022909](#) [55], nucleotides 210473 to 221016, loci *T285_RS00860* to *T285_RS00915*); DPC6026 (GenBank accession number [NC_017477](#) [56], nucleotides 202698 to 210932, loci *LJP_RS00920* to *LJP_RS00960*); NCC533 (GenBank accession number [NC_005362](#) [57], nucleotides 196136 to 202659, loci *LJ_RS00845* to *LJ_RS00880*), and Byun-jo-01 (GenBank accession number [NZ_CP029614](#) [D. Kim, unpublished], nucleotides shown in complement 1111505 to 1117990, loci *C0060_RS05265* to *C0060_RS05300*) with the *GtrA*-*GtrB* pairs aligned. (B) Nucleotide alignment of the sequences in panel A performed with Mauve to indicate areas of high sequence conservation. HicB, Hic B family antitoxin; phage tail, putative phage tail-related protein; HP, hypothetical protein; 30S, 30S ribosomal protein S14; MFS transporter, major facilitator family transporter; sug-trans, sugar transporter.

and mouse feces (Byun-jo-01), selecting the area between homologues of 2,3-diphosphoglycerate-dependent phosphoglycerate mutase and an aldose 1-epimerase family protein (Fig. 5). The conservation of ORFs surrounding the *gtrAB* pair varies among strains, with some ORFs being present but interrupted by stop codons. The section encoding the 30S ribosomal protein, 241 and 242, is present in all genomes. Translated sequences of ORFs which are present in more than one genome show high amino acid similarity between strains; the 242 sequence (NCBI reference sequence [WP_012845545](#)) shows 99 to 100% identity with the equivalent sequences in the other genomes (NCBI reference sequences [WP_012845545](#), [WP_011161379](#), and [WP_014567007](#)). Alignment of the surrounding nucleotide region showed high conservation of the region covering the 241-242 pair, and analysis of these two genes in the 6 genomes showed between 97.1 and 99.8% nucleic acid identity with the FI9785 sequence (Fig. 5B). The central region of strong nucleotide conservation stretches from upstream of the 30S ribosomal gene to the noncoding sequence after 242.

241 is required for EPS1 biosynthesis. To confirm the involvement of the putative flippase 241 in EPS1 production, a deletion mutant ($\Delta 241$) and its derivatives containing a 241 expression plasmid ($\Delta 241$ -p241) or an empty plasmid control ($\Delta 241$ -pQ10001) were constructed and their EPS analyzed using NMR. As with $\Delta 242$, gene deletion prevented EPS1 production while complementation restored biosynthesis (Fig. 6; Fig. S1C). A mutant where both 241 and 242 were deleted also showed production of EPS2 only (data not shown).

EPS1 production affects growth. The $\Delta 242$ and $\Delta 241$ strains both showed a slower growth phenotype than the wild type, both in liquid and on solid media (Fig. 7). This phenotype was similar when the strain contained an empty vector control, but normal growth was restored in liquid by overexpression of the 242 or 241 gene, although plate

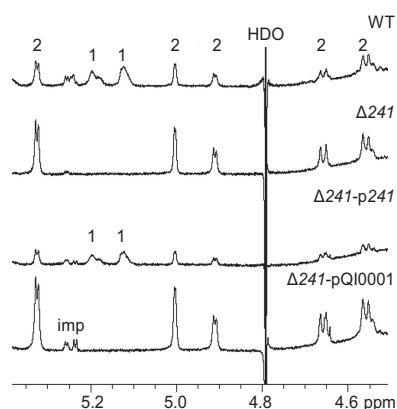


FIG 6 NMR analysis of pellet-associated EPS showing the effect of 241 deletion and complementation. The 600 MHz ^1H NMR spectra of EPS from WT and engineered *L. johnsonii* (pellet samples, D_2O , 300°K) are shown. Anomeric signals of EPS1 and EPS2 are labeled 1 and 2, respectively; imp, impurities.

growth remained slightly retarded in the 242 complemented mutant. Mutant colonies did reach the size of typical 1-day WT colonies after further incubation within 2 days. The slow-growth phenotype was maintained during growth in anaerobic conditions and at a lower temperature (30°C). The presence or absence of EPS1 did not seem to affect aggregation, while as noted previously, nonproduction of EPS2 in ΔepsE caused a strong aggregation phenotype (21), suggesting that EPS2 is a primary contributor to low aggregation of the WT (Fig. 7C). Deletion of 242 also did not have a strong effect on colony phenotype, with colonies retaining a rough and crinkled appearance, although overexpression of 242 resulted in a smoother colony upon longer incubation. Transmission electron microscopy (TEM) showed that the $\Delta 242$ and $\Delta 241$ mutants retained a visible EPS layer; this was more frequently irregular than in WT samples (Fig. 7E). Cells overexpressing 242 or 241 also exhibited a thick EPS layer, and in the case of $\Delta 242\text{p}242$, this layer was consistently paler, suggesting a different response to the osmium staining.

DISCUSSION

Effect of EPS2 loss on the *L. johnsonii* FI9785 proteomic profile. Apart from variations in proteins associated with ribosome structure, translation, and protein synthesis, very few biological processes seemed strongly affected in the soluble protein content by the loss of EPS2 synthesis in the $\Delta\text{eps_cluster}$ mutant strain. Comparative analysis of proteins from *Lactobacillus plantarum* grown at two temperature conditions, which gave a 10-fold difference in EPS production, also showed few changing proteins (29). It is interesting that loss of EPS2 production correlated with lower abundance of 242 in the $\Delta\text{eps_cluster}$ mutant strain than in the WT. We have now determined that this mutant is able to produce EPS1, but its biosynthesis is affected, either by the absence of the *eps* cluster genes or EPS2 itself or in response to changed cell conditions responding to reduction of a protective layer. The regulation of EPS synthesis has been linked to external signal and quorum sensing in a range of bacteria, including *L. plantarum* (30). BLAST analysis of a putative transcriptional regulator, D0R501, which was also less abundant in the $\Delta\text{eps_cluster}$ strain, showed a relationship to the YebC/PmpR family; regulators of this family are involved in a range of processes, including quorum sensing (31). Further investigation of the regulation of EPS1 and EPS2 genes, proteins, and polymers and how they relate to each other will be an interesting area for future study.

Involvement of putative flippase and bactoprenol glycosyltransferase in homopolysaccharide biosynthesis in *L. johnsonii*. The evidence from EPS NMR profiles from deletion and complementation strains indicates that putative bactoprenol glycosyltransferase 242 and neighboring putative flippase 241 are key components in the

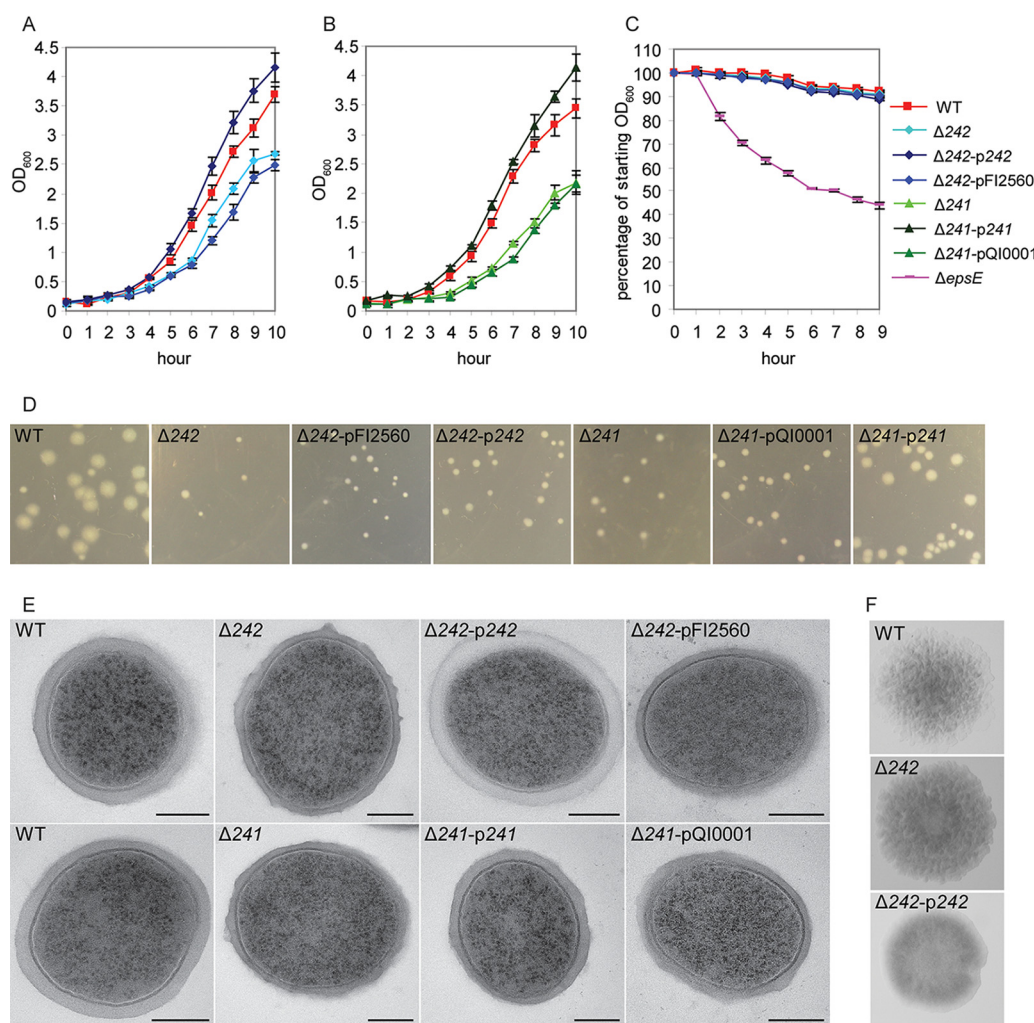


FIG 7 Phenotypic characterization of 241 and 242 deletion. (A and B) Growth of *L. johnsonii* strains in liquid at 37°C showing an increase in optical density. (C) Aggregation of overnight cultures. (D) Differences in colony size in strains given the same incubation time at 37°C. (E) TEM analysis of cells from overnight cultures (bar = 200 nm); WT, wild type. (F) Colony phenotypes.

production of the branched glucan EPS1. In lactic acid bacteria, α -glucans such as dextran are commonly synthesized by glucansucrases, which cleave sucrose and then add glucose to a growing chain (17). Three other mechanisms of EPS and O-antigen polysaccharide (O-PS) biosynthesis have been described in bacteria—the Wzx/Wzy-dependent pathway, the ATP-binding cassette (ABC) transporter-dependent pathway, and the synthase-dependent pathway (32). The first two mechanisms begin with the addition of a phosphorylated monosaccharide from a UDP-sugar to a lipid carrier, commonly thought to be undecaprenyl phosphate (5, 33, 34), while the synthase pathway utilizes cytosolic nucleotide-activated sugars (35, 36). Guan and coworkers described a three-gene operon, *gtrABX*, involved in O-antigen glycosylation in a bacteriophage infecting *Shigella flexnerii* and demonstrated that bactoprenol glucose transferase GtrB transferred [14 C]glucose to decaprenyl phosphate *in vitro* (28). They proposed a model where GtrB catalyzes the transfer of glucose from UDP-glucose to bactoprenol, GtrA flips the complex across the cytoplasmic membrane, and specific glycosyltransferase GtrX transfers the glucose to a specific residue on the O-antigen repeating unit (28). More recently, GtrB homologues have been shown to be involved in glycosylation of lipoteichoic and wall teichoic acids, and a similar 3-component mechanism has been proposed (37–39).

Our hypothesis is that 242 acts as a GtrB homologue, adding a glucose molecule to a lipid carrier, while the product of neighboring gene 241 functions as a flippase. However, the full process of chain and branch formation, and the possible involvement of glycosyltransferases elsewhere in the genome, remains to be determined. 241-242 may be involved in the decoration of a linear chain synthesized by other enzymes or may be an integral part of a biosynthetic cluster. The ability of bacterial glycosyltransferases to act on different substrates and even in different pathways has been noted (40). The genes encoding the three-component system involved in *Staphylococcus aureus* lipoteichoic acid glycosylation are not all located together on the chromosome (38), so it would not be unprecedented for a distant gene(s) to be involved in a three- or four-component EPS biosynthetic pathway. The genome of *L. johnsonii* F19785 contains several other glycosyltransferase genes which may be involved in synthesis of a linear chain, acting in concert with 241-242 to produce the final external EPS1. It is hoped that further examination of these genes will lead to a clearer model for the synthesis of this unusual EPS.

Effect of 242 or 241 deletion on *L. johnsonii*. Mutations affecting *L. johnsonii* F19785 EPS synthesis have been shown to affect aggregation, biofilm formation, adhesion to human HT29 cells and chicken gut explants, and resistance to stress, suggesting that EPS has a protective capacity (20, 21, 23, 41). We found that gene deletion of 242 or 241 slowed bacterial growth. The slow-growth phenotype is still seen at lowered temperatures or in the absence of oxygen, suggesting that it is not caused by increased sensitivity of cells to these conditions due to a reduction of the EPS layer. Further, removal of EPS2 did not seem to have the same effect, as the $\Delta eps_cluster$ mutant strain showed a similar growth rate to the wild type when grown for proteomic analysis. It has been noted that mutations which might prevent the release of undecaprenyl phosphate by blocking the full EPS biosynthetic process affect cell viability, either by reducing the amount of undecaprenyl available for other processes or by membrane destabilization in the presence of lipid intermediates (5, 42). However, it is not obvious why deletion of a protein proposed to glycosylate the lipid carrier might have a similar effect unless there are other components of EPS1 biosynthesis that might also interact with the carrier.

In conclusion, we found that a putative glycosyltransferase, 242, was less abundant in the $\Delta eps_cluster$ strain and that deletion of its gene prevented the accumulation of EPS1, while plasmid complementation restored production. *In silico* analysis indicated that 242 and its preceding gene, 241, show similarity to two members of a three-component system, *gtrABX*, shown to mediate O-antigen glycosylation in Gram-negative bacteria and, more recently, to be involved in teichoic acid glycosylation in Gram-positive species. Further deletion and complementation studies showed that 241 was also essential for EPS1 production. High conservation of nucleotide sequence with other *L. johnsonii* strains and the presence of analogous genes in other lactobacilli suggest that this might be part of a novel mechanism for homopolysaccharide EPS biosynthesis in Gram-positive bacteria. EPS/O-PS biosynthetic pathways have been studied in detail, but many questions remain unanswered, and new enzymes are still being discovered (43). Given the potential technological applications of EPS, there is significant interest in engineering novel forms (32), and their important roles in protection and biofilm formation make EPS biosynthesis a valid target for novel strategies to control pathogens. Further discovery of alternative mechanisms may give future opportunities to both understand and exploit bacterial EPS synthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. johnsonii* strains were grown as described previously (41) in homemade De Man Rogosa Sharpe medium (MRS) using 2% glucose as a carbon source at 37°C. *Lactococcus lactis* MG1614 (44) was grown in GM17 (Oxoid) at 30°C. Plasmids were selected and maintained using chloramphenicol (pFI2560 and pQI0001) at 7.5 $\mu\text{g ml}^{-1}$ or 5 $\mu\text{g ml}^{-1}$ and erythromycin (pG⁺host9) at 10 $\mu\text{g ml}^{-1}$ and 5 $\mu\text{g ml}^{-1}$ for *L. johnsonii* and *L. lactis*, respectively. The *L. johnsonii* strains and plasmids produced and/or used in this study are listed in Table 2.

TABLE 2 *L. johnsonii* strains created and used in this study

| Strain | Genotype | Description | Plasmid | Reference |
|---------|----------------------|--|----------------------|------------|
| FI9785 | Wild type | Poultry isolate | | 18 |
| FI10754 | Δ eps_cluster | eps gene cluster deleted | | 20 |
| FI11504 | Δ 242 | FI9785 with 242 gene deleted | | This study |
| FI11646 | Δ 242-p242 | FI11504 complemented with the 242 gene in expression plasmid pFI2560 | pFI2843 | This study |
| FI11647 | Δ 242-pFI2560 | FI11504 with pFI2560 empty vector control | pFI2560 | This study |
| FI11669 | Δ 241 | FI9785 with 241 gene deleted | | This study |
| FI11670 | Δ 241-p241 | FI11669 complemented with the 241 gene in expression plasmid pQI0001 | pQI0002 | This study |
| FI11671 | Δ 241-pQI0001 | FI11669 with pQI0001 ^a empty vector control | pQI0001 ^a | This study |
| FI10785 | Δ epsA | epsA transcriptional regulator from eps gene cluster deleted | | 23 |
| FI10844 | Δ epsE | epsE priming glycosyltransferase from eps gene cluster deleted | | 21 |

^aPlasmid pFI2560 with cloning site NcoI altered to NdeI-BamHI.

Isolation of proteins. Soluble protein extracts were prepared from *L. johnsonii* FI9785 and Δ eps_cluster strains inoculated from overnight cultures at 2% into prewarmed medium and grown to an optical density (OD₆₀₀) of 2.0 (6 to 7 h). Cells from 15-ml aliquots were harvested by centrifugation at 3,000 \times g for 15 min at 4°C, washed with 5 ml phosphate-buffered saline (PBS) containing 1 \times cComplete protease inhibitor (Roche), recentrifuged, washed with 1 ml PBS/protease inhibitor, and recentrifuged at 13,000 \times g for 2 min at 4°C before removal of the supernatant and freezing on dry ice. Three biological replicates and one technical replicate were prepared for each strain. Pellets were resuspended in 500 μ l extraction buffer (50 mM HEPES [pH 7.7], 0.3% SDS, 1 \times protease inhibitor, 5 U ml⁻¹ RNase-free DNase [Promega], 10 mM MgSO₄, and 1 mM CaCl₂) and then sonicated using a Soniprep 150 (Sanyo) for 7 cycles of 15 s with 30 s incubation on ice between cycles. After centrifugation at 13,000 \times g for 25 min at 4°C to pellet debris, the supernatant was precipitated overnight with 5 volumes of cold acetone at -20°C. Proteins were collected by centrifugation at 14,000 \times g for 10 min at 4°C and stored at -20°C. Total soluble protein was resuspended in 250 μ l 0.5 M trimethylammonium bicarbonate buffer (Sigma), 0.05% SDS, and 1 \times protease inhibitor and stored in LoBind tubes (Eppendorf). Concentrations were measured using Bradford reagent (BioLine).

Quantitative mass spectrometry. Bacterial protein samples, three biological replicates of mutants and controls and one technical replicate, were digested by trypsin, and the tryptic peptides were labeled using the iTRAQ 8-plex kit (AB Sciex Pte. Ltd., USA) following the manufacturer's instructions. The samples of each experiment were pooled and fractionated using a high-pH reversed-phase peptide fractionation kit (Pierce, Thermo Fisher Scientific). Each single fraction was analyzed using an nLC MS/MS Orbitrap Fusion trihybrid mass spectrometer coupled with a nano flow ultrahigh-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific). The peptides were separated after being trapped on a C₁₈ precolumn, using a gradient of 3 to 40% acetonitrile in 0.1% formic acid over 50 min at a flow rate of 300 nl min⁻¹ at 40°C. The peptides were fragmented in the linear ion trap by a data-dependent acquisition method, selecting the 40 most intense ions. For label-free experiments, each tryptic peptide sample was analyzed in triplicate as described above. All analyses were performed in triplicate. The raw data were analyzed with MaxQuant version 1.6.2.3 (Resource Identification Portal [RRID]:SCR_014485) using Andromeda software and consulting the Uniprot_ *Lactobacillus johnsonii* (strain FI9785) (1,726 sequences) protein database; the tolerance on parents was 10 ppm and on fragments was 0.02 ppm. The variable modifications allowed were oxidation on methionine and carboxyamidomethylation on cysteine as fixed modifications. The false discovery rate was below 1% using a decoy and reverse database, and the identified proteins contained at least 2 peptides with at least 6 amino acids sequenced. iTRAQ and label-free quantitative analyses were also performed using MaxQuant software and evaluated using Perseus statistical software (RRID:SCR_015753) with a two-sided *t* test, setting a *P* value of less than 0.05 and false-discovery rate (FDR) less than 0.01. Gene Ontology analyses were performed using the QuickGO algorithm European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI; RRID:SCR_004608).

Plasmid construction and gene deletion. Genes were deleted from the *L. johnsonii* FI9785 chromosome as described previously (21) using the thermosensitive vector pG+host9 (45) containing a knockout cassette of the partial upstream and downstream genes, amplified and joined by splice overlap extension PCR using primers designed to generate restriction sites for cloning and to create spliced products (see supplemental text and Table 3). Initial cloning was performed using electrocompetent *Lactococcus lactis* MG1614 (46) with growth at 28°C. After sequence confirmation, plasmids were transformed into electrocompetent *L. johnsonii* FI9785 (47), and gene replacement was performed as described previously (45) using 30°C as the permissive temperature and 42°C as the nonpermissive temperature. For recovery of Δ 242 and Δ 241, it was necessary to recover deletions at 30°C, and excised plasmids were cured by successive subculturing. For complementation, the 242 gene was cloned into the *L. johnsonii* expression plasmid pFI2560 (21), and the 241 gene was cloned into pFI2560-derivative pQI0001 (see supplemental text); the ligation products and control vector were transformed into electrocompetent *L. johnsonii* FI9785 as before.

Bioinformatic analysis. Translated gene sequence homologies and domain searches were performed using Blastp (RRID:SCR_001010) (48). The *L. johnsonii* genome FN298497 (49) was reanalyzed using RAST (RRID:SCR_014606) (50). Amino acid alignments were performed using the clustalW algorithm (RRID:SCR_002909) in Vector NTI (Invitrogen; RRID:SCR_014265) and visualized using GeneDoc. Nucleo-

TABLE 3 Oligonucleotide primers used for creation of deletion constructs and plasmids and assessment of sequence integrity, integration, and excision

| Primer | Sequence 5'–3' ^a |
|----------------|---|
| 241Eco_F | GATGAATTCACGCTGCTTAG |
| 241splice243_R | CGGCTTTTTCATAT ACTTTAACAGTCTTTCTTAT |
| 243Spe_R | CTACTAGTCATGATTGATTTTGGT |
| 243splice241_F | AGAAAGACTGTTAAAGT ATATGACAAAAAGCCGA |
| 241_IF | GCTTCTACGTCACAGCTTCT |
| 243_IR | TCCACAGTTTCGAACTGGTG |
| 240_F | ATGTCTAAAGTGTGACTATATGTT |
| 240splice242_R | TACTTTAACAGTCTTTCTTA GGCTTATTTCCCTTCT |
| 242splice240_F | AGAAGGGAAAATAAGCCTA AGAAAGACTGTTAAAGTA |
| 242Spe_R | CATTGACTAGTCATCATTCGGTAGTC |
| 240_IF | GAATGTCTAAAGTGTGACTATATGTT |
| 242_IR | ACGGTTGTATTCAGGCATATTC |
| pGhost1 | AGTCACGACGTTGTAAAACGACG |
| pGhostR | TACTACTGACAGCTTCCAAGG |
| pForVec | ACAGCAATGTTACAAGTTGAAAT |
| p181 | GCGAAGATAACAGTGACTCTA |
| 242_COD2F | AAAAAATTATCAATTATAGTTCCTTG |
| 242_C_R | GAAGCTCCACGTGAACCTC |
| 241_NdeF | TAACATAT GGGTATTTTTAAAGAATAC |
| 241_BamR | TTTGGATCCTTTA ACAGTCTTTCTATTAC |

^aMismatching base pairs to insert restriction sites or for splice overlap extension are in bold.

tide alignments were performed using Geneious (Biomatters Ltd., New Zealand; RRID:SCR_010519); short sequences were aligned using Geneious alignment, and larger genome segments were aligned using Mauve (RRID:SCR_012852) (51).

Isolation and NMR spectroscopy of EPS. Crude EPS was isolated from 2-day 500-ml cultures grown in MRS at 37°C as described previously (21), except that the initial extraction of capsular EPS from the washed bacterial pellet was performed by sonication in 50 ml 1 M NaCl for 7 cycles of 45 s with 30 s incubation on ice between cycles, followed by centrifugation at 6,000 × *g* and 4°C for 30 min to remove bacterial debris before the rounds of ethanol precipitation, the initial ethanol precipitation was for 3 days instead of overnight, and crude EPS was not further purified by TCA precipitation. EPS samples were analyzed using NMR as before (20) but with heating to 338°K (Δ242 series) and an increased number of scans (1,024). Samples in the Δ241 series were measured at 300°K.

Growth, aggregation, and phenotype studies. Overnight (15-h) cultures of WT, Δ*epsE*, Δ242-p242, and Δ241-p241 strains and 20-h cultures of Δ242, Δ242-pFI2560, Δ241, and Δ241-pQI0001 strains were used as inocula for growth and aggregation studies. For liquid growth, 20-ml broths were inoculated at 2%, and the OD₆₀₀ of 10-fold diluted samples was measured every hour during aerobic growth at 37°C. Colony size on plates was monitored aerobically at 30°C and 37°C and anaerobically at 37°C. All liquid growth of plasmid-containing strains was supplemented with chloramphenicol, while plate growth was nonselective. For aggregation, triplicate 1-ml samples from vortexed overnight cultures were transferred to cuvettes, and the OD₆₀₀ was measured hourly during incubation at room temperature. Growth and aggregation assays were each performed three times, and representative curves are shown. TEM images were taken from overnight cultures as described previously (20).

Data availability. All strains reported in this work are deposited in the Quadram Institute Bioscience culture collection and are available from the corresponding author upon request. For accession numbers, see Table 2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

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M.J.M., A.D., and A.N. contributed to the conception and design of the study; M.J.M.,

A.D., I.J.C., and G.L.G. performed the acquisition, analysis, and interpretation of the data; all authors contributed to the writing of the manuscript.

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