Heterologous Expression of a Position 2-Substituted (1→3)-β-D-Glucan in Lactococcus lactis

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Exopolysaccharides play an important role in the rheology and texture of fermented foods, and among these β-glucans have immunomodulating properties. We show that the overproduction of the Pediococcus parvulus gtf glycosyltransferase in an uncapsulated Lactococcus lactis strain results in synthesis and secretion (300 mg liter−1) of a position 2-substituted (1→3)-β-D-glucan that has potential use as a food additive.

A variety of bacteria, animals, and plants produce extracellular polysaccharides (EPS) and capsular polysaccharides. Some EPS have industrial applications as gelling and emulsifying agents (21). Three bacterial EPS (xanthan, gellan, and curdlan) have been approved as food additives by the U.S. Food and Drug Administration, and EPS-producing lactic acid bacteria (LAB) are frequently used to improve texture and taste of dairy products (9). In addition, the (1→3)-β-D-glucan can promote antitumor and antimicrobial activity, by activating macrophages, other white blood cells, or dendritic cells (3). (1→3)-β-D-Glucans (1,3)-β-D-glucans with branched or cyclic structures, or (1→3)-β-D-glucans (15). The immune response to the glucans from eukaryotes (either linear or with 1→6 branches) and to the prokaryotic linear curdlan, used for making functional foods (tofu), has been characterized, and its activity has been correlated with their chemical structure, molecular weight and conformation (2, 15). However, the immunomodulating properties of the (1→3), (1→2)-β-D-glucans have not been reported. The Pediococcus parvulus (formerly Pediococcus damnosus) 2.6 strain synthesizes this type of EPS (7), and analysis of this and another (1→3), (1→2)-β-D-glucan-producing LAB strains showed that all carried the gtf gene (25). Its product, the GTF glycosyltransferase (hereafter referred to as GTF), belongs to the COG1215 membrane-bound glycosyltransferase family, it has glycosyltransferase activity and has identity (33%) only with the Ts1 glycosyltransferase of Streptococcus pneumoniae serotype 37 (12). This enzyme catalyzes the biosynthesis and secretion of this organism’s capsule (13), which is a β-D-glucan similar to the EPS synthesized by Pediococcus, and anti-serotype 37 antibodies agglutinate S. pneumoniae strains that over-express gtf (25).

We constructed plasmid pNGTF (see the supplemental material) in order to express the plasmidic P. parvulus gtf in L. lactis NZ9000 (MG1363 pepN:nisRK) (11), which allows inducible expression from PnisA by the addition of nisin to the growth medium. The plasmid carried the translational fusion PnisA:SDnisA:his tag:gtf and encoded GTF*, a mutant protein (GTF with an MSH10D4KA amino-terminal tag). To determine whether the GTF* protein encoded by NZ9000(pNGTF) had glycosyltransferase activity, the protein was overexpressed and tested bound to the cell membrane. Exponential cultures (optical density at 660 nm [OD660] = 0.6) of the GTF* over-expressor (PnisA-gtf) and the control strain NZ9000 carrying the vector plasmid pNZ8048 (PnisA) were grown in ESTY medium ( Pronadisa, Madrid, Spain) supplemented with 0.5% glucose and chloramphenicol (5 μg ml−1), and expression from the PnisA promoter carried by the strains was induced with 0.25 ng of nisin ml−1 and 1 h of incubation at 37°C. Membrane preparations from three independent cultures each of NZ9000 (pNGTF) and of NZ9000(pNZ8048) were obtained with UDP-[14C]glucose as previously described (25) with the following modifications: cells were treated with lysozyme (10 mg ml−1) for 30 min at 30°C before mechanical disruption, and then GTF* membrane-bound protein was sedimented by centrifugation at 349,000 × g for 26 min at 4°C. The glycosyltransferase activities present in the membrane preparations were tested with UDP-[14C]glucose as previously described (25). One unit of glycosyltransferase activity was expressed as the enzyme concentration which catalyzes the incorporation into a macromolecular product of 1 pmol of glucose per mg of total proteins per min. The level of glycosyltransferase activity detected in the GTF* overexpressor (38 ± 6 U mg of total protein−1) was 54-fold greater than that of the control strain (0.7 ± 0.2 U mg of total protein−1).

Production of EPS by induced cultures of NZ9000(pNGTF) was examined under the microscope (Fig. 1). Agglutination of the cultures, as revealed by phase-contrast microscopy (as previously described [25]), showed that immunoprecipitation of NZ9000(pNGTF) occurred with antibodies to pneumococcal 37 serotype (Fig. 1A). As expected, these antibodies did not react with NZ9000(pNZ8048) (Fig. 1B). Similarly, EPS was observed by electron microscopy in NZ9000(pNGTF) cultures (Fig. 1C) but not in NZ9000(pNZ8048) (Fig. 1D). After 24 h of
induction, NZ9000(pNGTF) cells retained a normal morphology (Fig. 1B versus Fig. 1D), cell lysis was not detected (result not shown), and most of the EPS appears not to be attached to the cells. These results indicate that expression of GTF\* in *L. lactis* confers the ability to synthesize and secrete the EPS. The EPS released to the medium by NZ9000(pNGTF) was quantified, purified, and structurally characterized. Cultures of the GTF\* overexpressor were grown and induced in CDM defined medium (20) (initial pH 7.0, not controlled) supplemented with 0.8% glucose. When the cultures reached an OD\textsubscript{660} of 0.6, the cells were concentrated twofold by centrifugation and resuspension in fresh medium (to reduce the toxic effect of lactic acid produced during growth) (16). Then, induction was triggered by addition of nisin at 0.25 ng ml\textsuperscript{-1}. After 1 h of induction at 37°C, the GTF\* overexpressor and control cultures reached an OD\textsubscript{660} of 2.2 ± 0.04, which remained unchanged even after 120 h of incubation (OD\textsubscript{660} = 2.1 ± 0.08). The levels of polysaccharides in the growth medium were analyzed at different times of induction (Fig. 2). Culture samples were heated (60°C, 20 min), and cells were removed by centrifugation (13,600 × g, 10 min, 4°C). After the addition of 2 volumes of ethanol to the supernatants, the EPS were precipitated overnight at −20°C and sedimented by centrifugation (13,600 × g, 20 min, 4°C). The precipitates were twice dispersed in aqueous 80% ethanol, sedimented as described above, and then finally dissolved in Milli-Q water. The total amount of carbohydrates

![FIG. 1. Detection of EPS production by microscopy. *L. lactis* NZ9000 carrying the indicated plasmids were grown in ESTY broth (Pronadisa) and induced with nisin as indicated in the text. (A and B) After 1 h of induction, cells were subjected to agglutination tests with *S. pneumoniae* type 37-specific antisera (Statens Seruminstitut, Dinamarca) and detection by contrast-phase microscopy, as previously described (25). (C and D) After 24 h of induction, cells were analyzed in a JEOL 1230 transmission electron microscope operated at 100 kV. Glow-discharged carbon-coated Formvar grids were placed facedown over a droplet of each culture concentrated threefold in 0.1 M ammonium acetate (pH 7). After 1 min, the grid was removed, blotted briefly with filter paper and, without drying, negatively stained with 2% uranyl acetate for 40 s and then blotted quickly and air dried.](http://aem.asm.org/)

![FIG. 2. Analysis of EPS production by *L. lactis* NZ9000(pNGTF). Lactococcal strains carrying either pNGTF (●) or pNZ8048 vector (○) were grown in modified CDM medium. At the indicated times of induction, samples were withdrawn, and the concentrations of polysaccharides present in the supernatants were determined (see details in the text). The values presented are the means of three independent cultures of each strain and in each condition.](http://aem.asm.org/)
in the EPS was determined by using the phenol-sulfuric acid method (6) with glucose as a standard. The production and secretion of the polysaccharide by NZ9000(pNGTF) increased during the first 48 h of induction, and then its levels in the supernatants (approximately 300 mg liter \(^{-1}\)) remained almost constant even after 120 h of incubation (Fig. 2 and results not shown), indicating that the EPS is either not degraded by \(L.\) lactis glycosidases or exists in an equilibrium between synthesis and degradation. As expected, only basal levels of polysaccharides were detected in supernatants of the control strain (Fig. 2).

The EPS of NZ9000(pNGTF) was purified from the supernatant of 48 h-induced-cultures. The EPS was subjected to three cycles of precipitation with 3 volumes of 100% cold ethanol, each one for 18 h at 4°C (first cycle), and then at \(-20°C\) (subsequent cycles) with sedimentation by centrifugation. The final precipitate was resuspended in water, dialyzed (molecular mass cutoff, 12,000 Da) against water, and lyophilized. Determination of the total neutral sugar content of the EPS by the phenol-sulfuric acid method showed the purity to be \(>90\%\) (correlation of weight with sugar concentration).

The neutral sugar composition was characterized by hydrolysis of the purified EPS with 3 M trifluoroacetic acid (1 h, 121°C). The hydrolysis products were reduced with sodium borohydride, and the resulting alditols were acetylated with pyridine-acetic anhydride (1:1, 1 h, 100°C). Identification and quantification were carried out by gas-liquid chromatography using an SP-2380 column (30 m by 0.25 mm, 0.2-\(\mu\)m film thickness), a temperature program (210 to 240°C, 3-min initial hold, 15°C min \(^{-1}\) ramp rate, and 7-min final time), and a flame ionization detector. This analysis for neutral sugars showed that the EPS was composed exclusively of glucose.

Infrared (IR) spectra were obtained by the KBr technique (18) using a Bruker IFS 28 FT-IR spectrophotometer. The IR spectrum of the EPS was characteristic of a \(\beta\)-glucan, displaying an absorption band at around 890 cm \(^{-1}\), which is characteristic of \(\beta\)-glycosidic linkages. In addition, the IR spectrum lacked bands at 850 and 930 cm \(^{-1}\) (typically attributed to \(\alpha\)-glucans), at 1,560 and 1,650 cm \(^{-1}\) (due to the CO-NH linkage of proteins or amino sugars), and at 1,730 to 1,750 cm \(^{-1}\) (due to the carboxylic group of uronic acids or esterified organic acids) (4, 10).

The linkage types between the glucose units were determined as follows. The EPS was methylated according to the method of Ciucanu and Kerek (5). The product was extracted with chloroform-methanol (1:1), dialyzed sequentially against water and then 50% ethanol, and evaporated. Methylation fractions, which showed negligible IR absorption for hydroxyl moieties, were hydrolyzed with 3 M trifluoroacetic acid (121°C, 1 h), and the products were reduced with NaBD\(_4\) and then acetylated and subjected to gas-liquid chromatography–mass spectrometry, using a SPB-1 column (30 m by 0.22 mm, 0.25-\(\mu\)m film thickness), a temperature program from 160 to 200°C (1-min initial hold and then a ramp rate of 2°C min \(^{-1}\)), and a mass detector Q-Mass (Perkin-Elmer, Norwalk, CT). Each component was quantified according to the peak area. The analysis identified partially methylated alditol acetates corresponding to terminal glucopyranose, 3-O-substituted glucopyranose, and 2,3-di-O-substituted glucopyranose (Table 1).

Thus, the results show that the EPS of \(L.\) lactis NZ9000 (pNGTF) is the same as that of \(P.\) parvulus 2.6 (7).

For molecular weight estimation, 10 mg of purified EPS were dissolved in 2 ml of a buffer (pH 7.3) consisting of 10 mM Na\(_2\)HPO\(_4\), 1 mM KH\(_2\)PO\(_4\), 440 mM NaCl, and 3 mM KCl. This preparation was centrifuged in a microfuge to eliminate insoluble material, and the supernatant passed through a column (60 by 2.6 cm) of Sepharose CL6B equilibrated with the same buffer (flow, 18 ml h \(^{-1}\)). The column had been previously calibrated with commercial standards (dextran blue, dextrans T70 and T10, and vitamin B\(_{12}\)). Fractions (3.5 ml) were recovered and monitored for carbohydrates using the phenol-sulfuric acid method. The EPS eluted as one peak (fractions 30 to 51), indicating that its average molecular mass was \(>1,000\) kDa, which is in the same range as that of the \(\beta\)-d-glucan purified from \(P.\) parvulus 2.6 (unpublished results).

In summary, expression of the \(P.\) parvulus GTF glucosyltransferase in \(L.\) lactis causes the production and secretion of 2-substituted-(1→3)-\(\beta\)-d-glucan. As far as we know, this is the first instance of the production of a \(\beta\)-glucan by \(L.\) lactis, although the production of \(\alpha\)-glucan dextran in a recombinant \(L.\) lactis strain by the expression of the \(L.\) mesenteroides dextranulclease DsD has been reported (17).

Expression in a defined medium, coupled with the purification method described here, allows ca. 80% recovery of pure \(\beta\)-d-glucan with a high molecular mass. It has been shown that \(\beta\)-glucans with masses \(>5\) kDa are biologically active, and they can act as \(\beta\)-glucan receptor antagonists (2). The \(\beta\)-d-glucan produced by NZ9000(pNGTF) will be tested in the future for its immunomodulator properties.

The \(P.\) parvulus 2.6 strain has been tested for the fermentation of oat-based products and produced an increase in the viscosity of the medium during the fermentation (14), indicating the potential utility of the position 2-substituted (1→3)-\(\beta\)-d-glucan as a thickening agent. The level of EPS produced by \(L.\) lactis NZ9000(pNGTF) (300 mg liter \(^{-1}\)) is similar to that reported for the parental producer strain \(P.\) damnosus 2.6 (120 mg liter \(^{-1}\)) in cultures grown without pH control (8). This level also falls within the range of homologous and heterologous EPS expression in \(L.\) lactis (50 to 500 mg liter \(^{-1}\)) (19), without the need of expressing gene clusters to synthesize and secrete the EPS (24) or (in some cases) regulatory proteins (19). The levels of EPS reported here could probably be improved by further optimization of fermentation conditions, as was the case for \(P.\) parvulus 2.6 (22). In vitro analysis of the GTF activity (25; the present study) and metabolic studies in \(P.\)
parvulus 2.6 (23) point to the central role of UDP-glucose as a key intermediate in the synthesis of β-D-glucan. The overexpression of the enzymes required for the synthesis of UDP-glucose should therefore result in increased levels of β-D-glucan in L. lactis as occurred in S. thermophilus (24). Consequently, L. lactis NZ9000(pNGTF) has the potential to be an excellent source of position 2-substituted (1→3)-β-D-glucan for analysis of its properties as a food additive.

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