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

María Laura Werning,[1] María Angeles Corrales,[1] Alicia Prieto,[1] Pilar Fernández de Palencia,[1] Jesús Navas,[2] and Paloma López[1]*

Departamento de Ciencia de Proteínas, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain,[1] and Departamento de Biología Molecular (Unidad Asociada al Centro de Investigaciones Biológicas, CSIC), Facultad de Medicina, Universidad de Cantabria, 39011 Santander, Spain[2]

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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-glucans can promote antitumor and antimicrobial activity, by activating macrophages, other white blood cells, or dendritic cells (3). (1→3)-β-D-Glucans occur as linear glucans, (1→3, 1→6)-β-D-glucans with branched or cyclic structures, or (1→3, 1→2)-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 their 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 other (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 glucosyltransferase activity and has identity (33%) only with the Tts 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 overexpress *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 P_{nisA} by the addition of nisin to the growth medium. The plasmid carried the translational fusion P_{nisA}-SD_{nisA}-his tag-*gtf* and encoded GTF*, a mutant protein (GTF with an $MSH_{10}D_4KA$ 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 [OD_{660}] = 0.6) of the GTF* overexpressor (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 NZ900(pNZ8048) were obtained tested with UDP-[^{14}C]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-[^{14}C]glucose as previously described (25). One unit of glucosyltransferase 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 glucosyltransferase 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 NZ900(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

* Corresponding author. Mailing address: Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain. Phone: 34-918373112. Fax: 34-915360432. E-mail: plg@cib.csic.es.

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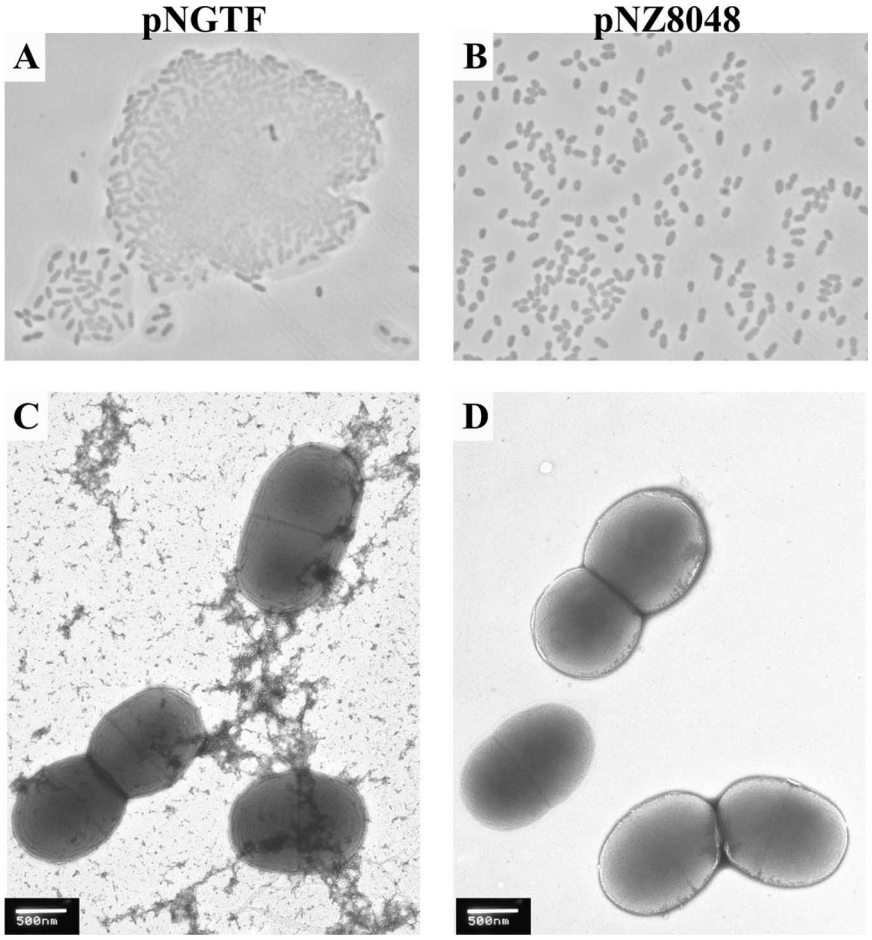

FIG. 1. Detection of EPS production by microscopy. *L. lactis* NZ9000 carrying the indicated plasmids were grown in ESTY broth (Pronadisa) and induced with nisin as indicated in the text. (A and B) After 1 h of induction, cells were subjected to agglutination tests with *S. pneumoniae* type 37-specific 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.

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_{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^{-1}. After 1 h of induction at 37°C, the GTF* overexpressor and control cultures reached an OD_{660} of 2.2 ± 0.04, which remained unchanged even after 120 h of incubation (OD_{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

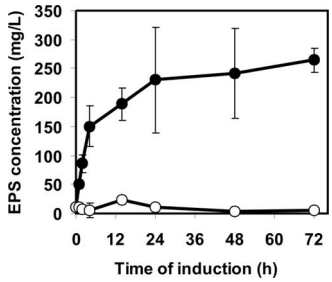

FIG. 2. Analysis of EPS production by *L. lactis* NZ9000(pNGTF). Lactococcal strains carrying either pNGTF (●) or pNZ8048 vector (○) were grown in modified CDM medium. At the indicated times of induction, samples were withdrawn, and the concentrations of polysaccharides present in the supernatants were determined (see details in the text). The values presented are the means of three independent cultures of each strain and in each condition.

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-μm 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 β-glucan, displaying an absorption band at around 890 cm^{-1}, which is characteristic of β-glycosidic linkages. In addition, the IR spectrum lacked bands at 850 and 930 cm^{-1} (typically attributed to α-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. Methylated fractions, which showed negligible IR absorption for hydroxyl groups, 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-μm 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).

TABLE 1. Percentages of the linkage types deduced from methylation analysis of the EPS produced by *L. lactis*

Retention time (min)	Linkage type	Characteristic fragments (*m*/*z*)	Relative abundance (%)
8.59	Glc*p*-(1→	87, 102, 118, 129, 145, 161, 162, 205	33
10.78	→3)-Glc*p*-(1→	101, 118, 129, 161, 173, 233	34
12.80	→2,3)-Glc*p*-(1→	87, 101, 129, 161, 202, 262	33

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_2HPO_4, 1 mM KH_2PO_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 β-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)-β-D-glucan. As far as we know, this is the first instance of the production of a β-glucan by *L. lactis*, although the production of α-glucan dextran in a recombinant *L. lactis* strain by the expression of the *Leuconostoc mesenteroides* dextransucrase DsrD has been reported (17).

Expression in a defined medium, coupled with the purification method described here, allows ca. 80% recovery of pure β-D-glucan with a high molecular mass. It has been shown that β-glucans with masses >5 kDa are biologically active, and they can act as β-glucan receptor antagonists (2). The β-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)-β-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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REFERENCES

1. Reference deleted.
2. **Brown, G. D., and S. Gordon.** 2005. Immune recognition of fungal β-glucans. Cell. Microbiol. **7:**471–479.
3. **Brown, G. D., and S. Gordon.** 2001. A new receptor for β-glucan. Nature **413:**36–37.
4. **Cerna, M., A. S. Barros, A. Nunes, S. M. Rocha, I. Delgadillo, J. Copikova, and M. A. Coimbra.** 2003. Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. Carbohydr. Polymers **51:**383–389.
5. **Ciucanu, I., and F. Kerek.** 1984. A simple and rapid method for the permethylation of carbohydrates. Carbohydr. Res. **131:**209–217.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric methods for determination of sugars and related substances. Anal. Chem. **28:**350–356.
7. **Dueñas-Chasco, M. T., M. A. Rodriguez-Carvajal, P. T. Mateo, G. Franco-Rodriguez, J. Espartero, A. Irastorza-Iribas, and A. M. Gil-Serrano.** 1997. Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6. Carbohydr. Res. **303:**453–458.
8. **Dueñas, M., A. Munduate, A. Perea, and A. Irastorza.** 2003. Exopolysaccharide production by *Pediococcus damnosus* 2.6 in a semidefined medium under different growth conditions. Int. J. Food Microbiol. **87:**113–120.
9. **Jolly, L., S. J. F. Vincent, P. Duboc, and J. R. Neeser.** 2002. Exploiting exopolysaccharides from lactic acid bacteria. Antonie van Leeuwenhoek **82:**367–374.
10. **Kacurakova, M., and R. H. Wilson.** 2001. Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. Carbohydr. Polymers **44:**291–303.
11. **Kuipers, O. P., P. G. G. A. de Ruyter, M. Kleerebezem, and W. M. de Vos.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:**15–21.
12. **Llull, D., E. Garcia, and R. Lopez.** 2001. Tts, a processive β-glucosyltransferase of *Streptococcus pneumoniae*, directs the synthesis of the branched type 37 capsular polysaccharide in pneumococcus and other gram-positive species. J. Biol. Chem. **276:**21053–21061.
13. **Llull, D., R. Munoz, R. Lopez, and E. Garcia.** 1999. A single gene (*tts*) located outside the *cap* locus directs the formation of *Streptococcus pneumoniae type* 37 capsular polysaccharide: type 37 pneumococci are natural, genetically binary strains. J. Exp. Med. **190:**241–252.
14. **Mårtensson, O., M. Dueñas, A. Irastorza, R. Ošte, and O. Holst.** 2003. Comparison of growth characteristics and exopolysaccharide formation of two lactic acid bacteria strains, *Pediococcus damnosus* 2.6 and *Lactobacillus brevis* G-77, in an oat-based, nondairy medium. Lebensm.-Wiss. Technol. **36:**353–357.
15. **McIntosh, M., B. A. Stone, and V. A. Stanisich.** 2005. Curdlan and other bacterial (1-3)-β-glucans. Appl. Microbiol. Biotechnol. **68:**163–173.
16. **Mierau, I., K. Olieman, J. Mond, and E. Smid.** 2005. Optimization of the *Lactococcus lactis* nisin-controlled gene expression system NICE for industrial applications. Microb. Cell Factories **4:**16.
17. **Neubauer, H., A. Bauché, and B. Mollet.** 2003. Molecular characterization and expression analysis of the dextransucrase DsrD of *Leuconostoc mesenteroides* Lcc4 in homologous and heterologous *Lactococcus lactis* cultures. Microbiology **149:**973–982.
18. **Price, W. J.** 1972. Sample handling techniques, p. 97–128. *In* R. G. J. Miller and B. C. Stace (ed.), Laboratory methods in infrared spectroscopy. Heyden and Son, London, England.
19. **Ruas-Madiedo, P., and C. G. de los Reyes-Gavilan.** 2005. Invited review: methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J. Dairy Sci. **88:**843–856.
20. **Sánchez, C., A. R. Neves, J. Cavalheiro, M. Moreira dos Santos, N. García-Quintáns, P. López, and H. Santos.** 2008. The contribution of citrate metabolism to the growth of *Lactococcus lactis* CRL264 at low pH. Appl. Environ. Microbiol. **74:**1136–1144.
21. **Sutherland, I. W.** 2001. Microbial polysaccharides. Int. Dairy Res. **11:**663–674.
22. **Velasco, S., E. Arskold, M. Paese, H. Grage, A. Irastorza, P. Radstrom, and E. W. J. van Niel.** 2006. Environmental factors influencing growth of and exopolysaccharide formation by *Pediococcus parvulus* 2.6. Int. J. Food Microbiol. **111:**252–258.
23. **Velasco, S. E., M. J. Yebra, V. Monedero, I. Ibarburu, M. T. Dueñas, and A. Irastorza.** 2007. Influence of the carbohydrate source on β-glucan production and enzyme activities involved in sugar metabolism in *Pediococcus parvulus* 2.6. Int. J. Food Microbiol. **115:**325–334.
24. **Welman, A. D., and I. S. Maddox.** 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. **21:**269–274.
25. **Werning, M. L., I. Ibarburu, M. T. Dueñas, A. Irastorza, J. Navas, and P. López.** 2006. *Pediococcus parvulus gtf* gene encoding the GTF glycosyltransferase and its application for specific PCR detection of β-D-glucan-producing bacteria in foods and beverages. J. Food Prot. **69:**161–169.