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Probiotic Properties of the 2-Substituted (1,3)-β-D-Glucan-Producing Bacterium *Pediococcus parvulus* 2.6▽

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Exopolysaccharides have prebiotic potential and contribute to the rheology and texture of fermented foods. Here we have analyzed the in vitro bioavailability and immunomodulatory properties of the 2-substituted (1,3)-β-D-glucan-producing bacterium *Pediococcus parvulus* 2.6. It resists gastrointestinal stress, adheres to Caco-2 cells, and induces the production of inflammation-related cytokines by polarized macrophages.

Lactic acid bacteria (LAB) are industrially important microorganisms for fermented food production. The recent widespread application of LAB and bifidobacteria for elaboration of functional food is attributable to the accumulating scientific evidence showing their beneficial effects on human health (3, 16). Most commercialized probiotics are limited to a few strains of bifidobacteria, lactobacilli, and streptococci, most of which produce exopolysaccharides (EPS) (27, 30). This fact, together with reports on immunomodulating ability as well as anticarcinogenic and cholesterol-lowering activities of EPS-producing LAB (25), suggests that the beneficial properties of these microorganisms for human health may be due to the biological activities of these prebiotic biopolymers (25, 26), whose producing bacteria are also frequently used to improve the texture and taste of dairy products (5, 11, 25). The future development of functional foods will be aimed at the diversification of this class of food, and therefore the identification and characterization of further bacteria with probiotic potential, isolated from habitats different from those of the currently used organisms (digestive tract and dairy products), will increase the biodiversity and utility of this class of microorganisms.

LAB strains belonging to the genera *Pediococcus*, *Lactobacillus*, and *Oenococcus*, isolated from cider and wine, produce a 2-substituted (1,3)-β-D-glucan EPS (4, 6, 7, 12, 17). One of these strains is *Pediococcus damnosus* 2.6 (also known as ropy and 2.6R), originally isolated from cider (8) and later renamed *Pediococcus parvulus* 2.6 (32). Curing of its 35-kDa pPP2 plasmid generated the isogenic, nonropy (2.6NR), non-EPS-producing strain (8). The plasmidic *gtf* gene determinant for EPS production was cloned into *Escherichia coli*, and determination of its DNA sequence revealed that it encodes a protein, named GTF glycosyltransferase, belonging to the COG1215 membrane-bound glycosyltransferase family (32). Cloning of the *gtf* gene and functional expression of its encoded glycosyltransferase in *Streptococcus pneumoniae* (32) and *Lactococcus lactis* revealed that this enzyme is indeed responsible for the synthesis of the β-D-glucan (33).

The GTF glycosyltransferase has identity (33%) only with the Tts glycosyltransferase of *Streptococcus pneumoniae* serotype 37 (19). The latter enzyme catalyzes the biosynthesis and secretion of this organism's capsule (18), which is a β-D-glucan similar to the EPS synthesized by *Pediococcus*, and anti-serotype 37 antibodies also agglutinate *Streptococcus pneumoniae* (32) and *Lactococcus lactis* strains that overexpress *gtf* (4, 33), as well as LAB strains naturally carrying this gene (4, 32).

Analysis of the rheological properties of the β-D-glucan synthesized by *P. parvulus* 2.6 showed that it has potential utility as a biothickener (29). In addition, human ingestion of oat-based food elaborated with *P. parvulus* 2.6 resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)-β-D-glucans in oats (21). Therefore, this LAB is a potential probiotic strain useful for elaboration of functional food.

In this work, we performed a comparative analysis of the β-D-glucan producer *P. parvulus* 2.6 and its isogenic nonropy strain, using in vitro models that simulate the conditions in the human gastrointestinal (GI) tract.

Cultures of the strains were grown to early stationary phase in MRS medium (Pronadisa, Madrid, Spain) at 30°C under anaerobic conditions. Aliquots containing 3.4×10^7 cells of each bacterium were independently subjected to agglutination tests with *S. pneumoniae* type 37-specific antiserum (Statens Serum Institut, Copenhagen, Denmark), as previously described (32), and production of EPS was examined under a microscope (Fig. 1). Agglutination of the cultures, detected by phase-contrast microscopy as previously described (33), showed that immunoprecipitation of strain 2.6R occurred with antibodies against pneumococcal serotype 37 (Fig. 1A). As expected, these antibodies did not react with strain 2.6NR (Fig. 1B). This type of analysis, coupled with plate counting, revealed that growth of *P. parvulus* 2.6 up to the beginning of the stationary phase was an optimal condition for EPS production without a loss of viability (results not shown). Therefore, the strains were grown to an optical density at 620 nm (OD_{620}) of 1.2 (10^9 CFU ml^{-1}) as described above and subjected to conditions of the human gut by use of an in vitro model which

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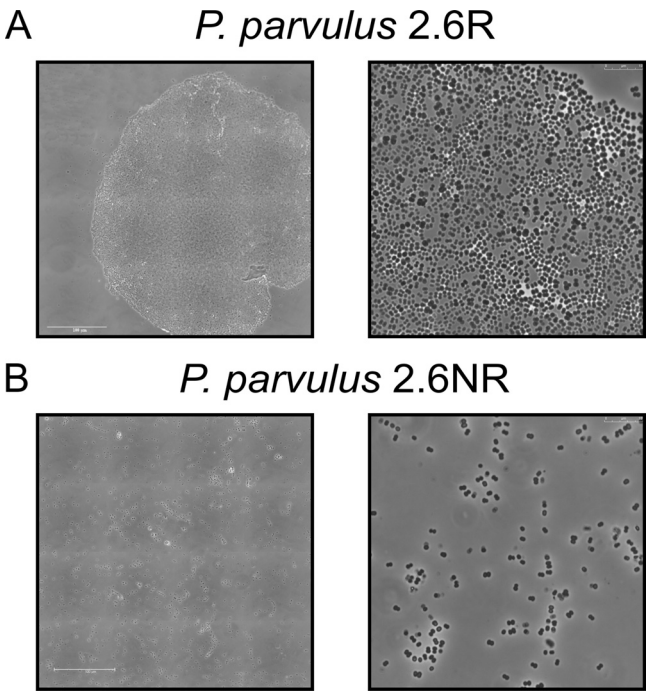

FIG. 1. Detection of EPS production. The *P. parvulus* 2.6R (A) and 2.6NR (B) strains were subjected to agglutination tests and detection by phase-contrast microscopy. Left panels, bar = 100 μm; right panels, bar = 10 μm.

approximates exposure to saliva, the pH gradient of the stomach, and intestinal stress (Fig. 2), as previously described (9), with the following modifications. For gastric (G) stress analysis, bacteria were treated for 20 min, after exposure to lysozyme, with pepsin at the following pHs: 5.0, 4.1, and 3.0. Moreover, GI stress was mimicked by exposure of the G, pH 5.0, samples to bile salts and pancreatin at pH 6.5 for 120 min. Treated bacteria (G and GI samples) were further analyzed for cell viability as previously described (9) and then compared with untreated bacteria (C samples) by using Live/Dead BacLight fluorescent stain, which permits the calculation of the percentage of live cells from the ratio of green (live) to red (dead) fluorescence. Since the presence of the EPS attached to the ropy strain could impair proper staining of the cells, prior to this analysis we established that (i) for both strains the green/red ratio correlates with the number of viable cells determined by plate counting and (ii) the dyes were taken up by *P. parvulus* 2.6 cells, as determined by fluorescence microscopy analysis (data not shown).

Figure 2 depicts the results of the analysis of *P. parvulus* 2.6 and 2.6NR subjected to G or GI stress. Both strains showed the same pattern of resistance to stress, indicating that the presence of EPS did not confer to *P. parvulus* 2.6 an advantage for survival in the human digestive tract. After exposure to pH 3.0, approximately 10% cell survival was detected for both strains. In addition, the intestinal conditions caused no marked loss of viability (GI, pH 5.0, versus G, pH 5.0, samples), indicating that live bacteria could be available for interaction with intestinal epithelial cells. This interaction was investigated by using human Caco-2 cells and a ratio of 10 bacteria per epithelial cell, as previously described (9). After 1 h of exposure to bacteria, the Caco-2 cells were washed three times with phosphate-buffered saline (PBS), pH 7.1, to remove unadhered bacteria, and then the Caco-2 cells were detached by treatment with 0.5% trypsin-EDTA (Invitrogen, Barcelona, Spain) and the number of adhered bacteria was determined by plate counting. In the control experiments, after 1 h of exposure to bacteria the Caco-2 cells were detached with trypsin, as described above, but without any washing, and were plate counted to determine the total number (i.e., adhered and unadhered) of bacteria. Results from the adhesion experiments are expressed as percentages of the corresponding control value.

In further experiments, two probiotic strains were used, namely, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* subps. *lactis* BB-12 (Chr. Hansen A/S, Hørsholm, Denmark), which had previously shown high and intermediate levels of adhesion (9). All bacteria were grown to early stationary phase in MRS medium as described above, sedimented by centrifugation at 12,000 × *g*, and used for adhesion experiments, after resuspension in PBS, pH 7.1, at 1.25×10^6 cells ml^{-1}. In addition, for analysis of the influence of the EPS on the adhesion capability of the ropy strain, two subpopulations were used, (i) prepared as indicated above (2.6R) and (ii) composed of bacterial cells washed with PBS prior to resuspension as described above (2.6R*), with the aim to remove

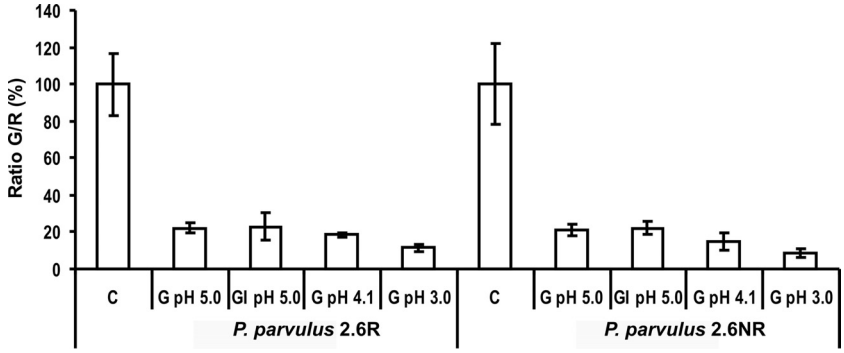

FIG. 2. Analysis of cell survival after G and GI stresses. The indicated bacterial strains were left untreated (C) or subjected to various G or GI stresses as described in the text. After staining, cell viability was analyzed by measurement of green and red fluorescence. The values are the means for three independent experiments and are expressed as percentages of the green/red (G/R) fluorescence ratio for untreated control samples. The 100% control values for untreated 2.6R and 2.6NR were 10.05 and 9.98, respectively.

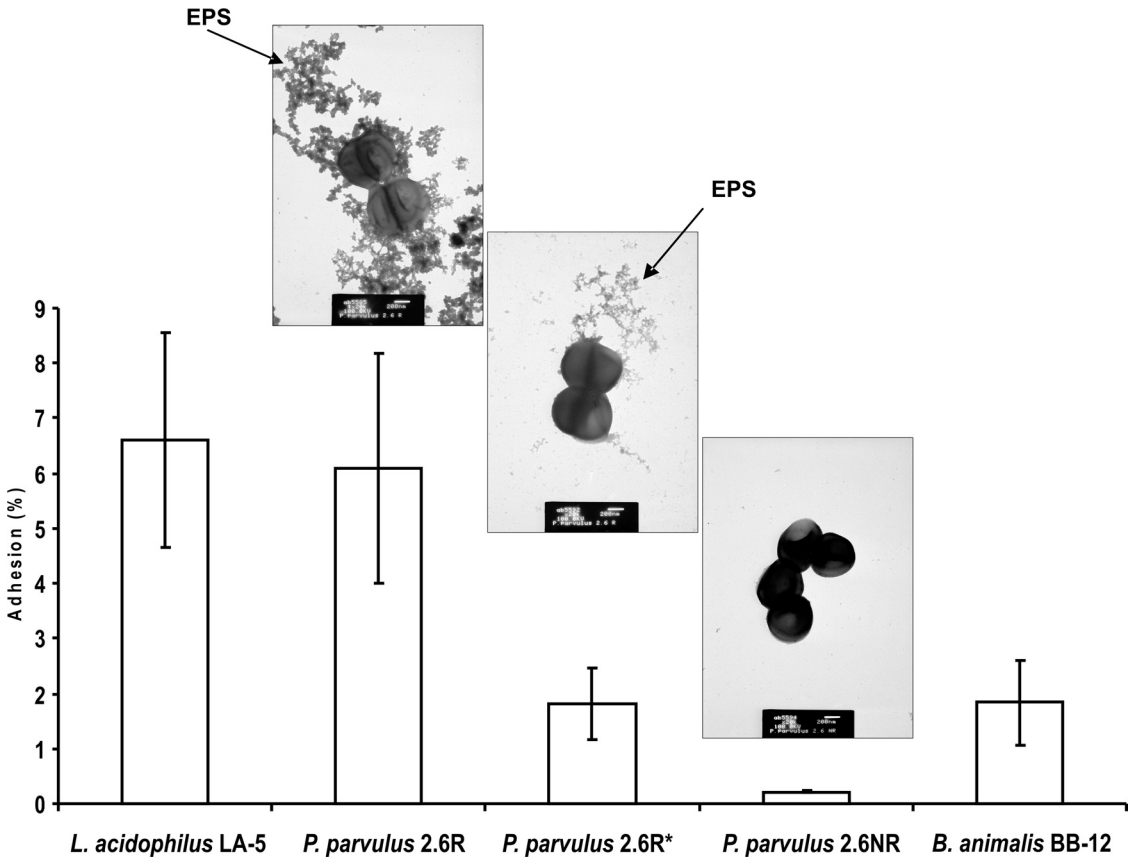

FIG. 3. Adhesion of bacterial strains to Caco-2 cells. Adhesion levels are expressed as percentages of the total number of bacteria (adhered plus unadhered) detected after exposure to Caco-2 cells for 1 h. Each adhesion assay was conducted in triplicate. The values are the means for three independent experiments in which three independent determinations were performed. (Insets) Prior to the adhesion experiments, bacteria were analyzed in a JEOL 1230 transmission electron microscope operated at 100 kV.

the EPS attached to bacteria before analyzing their adhesion. Prior to that, an analysis of the bacteria by electron microscopy was performed using samples prepared as follows. Glow-discharged carbon-coated Formvar grids were placed facedown over a droplet of each culture concentrated fivefold in 0.1 M $AcNH_4$, pH 7. After 1 min, each grid was removed, blotted briefly with filter paper, and without being dried, negatively stained with 2% uranyl acetate for 40 s and then blotted quickly and air dried. The analysis (Fig. 3) revealed that EPS bound to *P. parvulus* 2.6 was indeed present and was partially removed by the washing treatment. Moreover, the analysis confirmed the absence of EPS in the 2.6NR strain. Figure 3 depicts the results of the adhesion experiments. *P. parvulus* 2.6 showed a high level of adherence (6.1%), similar to that of *L. acidophilus* LA-5 (6.6%) and considerably higher than that of the non-EPS-producing strain 2.6NR (0.25%). In addition, an intermediate adherence (1.8%) was detected for the 2.6R* subpopulation of the 2.6R strain and for *B. animalis* BB-12. These results strongly supported a contribution of the EPS of *P. parvulus* 2.6 to attachment to colon epithelial cells. Therefore, the immunomodulatory properties of the 2.6R and 2.6NR strains on macrophages were investigated. To that end, proinflammatory M1 and anti-inflammatory M2 macrophages were generated from human peripheral blood mononuclear cells, using 1,000 U ml^{-1} granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor (10 ng ml^{-1}), as previously described (31), and their cytokine responses after exposure to the ropy and nonropy strains for 18 h were determined by means of enzyme-linked immunosorbent assay (34), using antibodies against tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8), and IL-10 (ELISA set; ImmunoTools, Friesoythe, Germany). With regard to proinflammatory cytokines, both bacterial strains induced high levels of TNF-α (Fig. 4A) and IL-8 (Fig. 4B) on M1 macrophages but had a minor (TNF-α) or absent (IL-8) effect on M2 macrophages. Both strains also induced the production of the anti-inflammatory cytokine IL-10, although the extent of cytokine release was higher in M2 macrophages (Fig. 4C). However, the levels of TNF-α and IL-8 produced by M1 macrophages were higher in response to the 2.6NR strain (Fig. 4A and B), thus implying that elimination of the pPP2 plasmid, which encodes the *P. parvulus* 2.6 EPS, triggers a higher level of proinflammatory cytokines in M1 macrophages. Although a contribution by other, unknown products encoded by pPP2 cannot be ruled out, these results strongly suggest that the presence of EPS in *P. parvulus* 2.6 counteracts the proinflammatory activation of M1 macrophages in response to the bacterium. Consequently, EPS might act by (i) preventing recognition by M1 macrophage-expressed Toll-like receptor 2 (TLR2) of

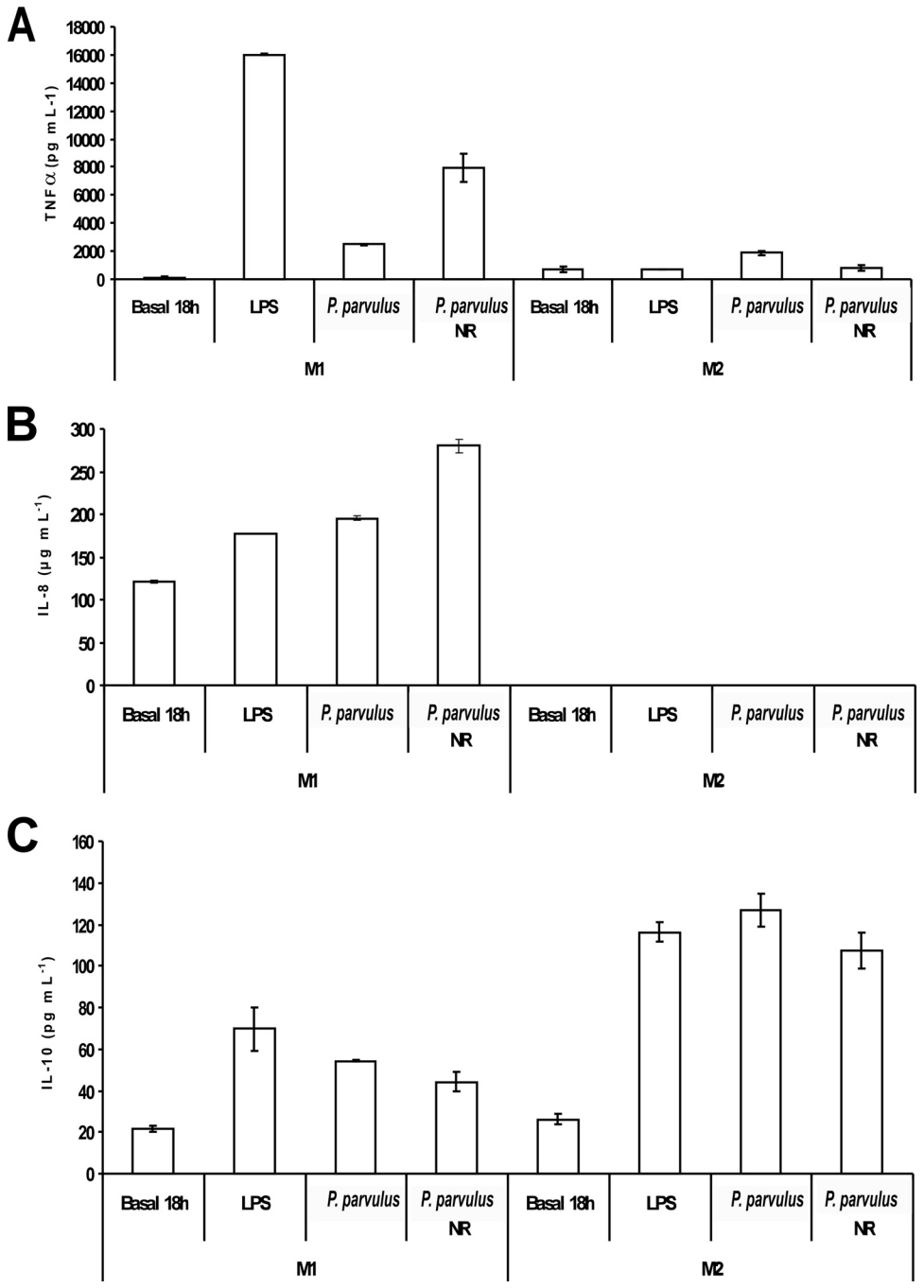

FIG. 4. Cytokine responses of macrophages to *P. parvulus* strains. M1 and M2 macrophages were either left untreated (basal 18 h) or stimulated with lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma, Barcelona, Spain) at 10 ng ml^{-1}, *P. parvulus* 2.6 (2.6R), or its nonropy mutant (2.6NR), and the levels of TNF-α (A), IL-8 (B), and IL-10 (C) released were determined. Each determination was performed in triplicate, and the means and standard deviations are shown.

the major gram-positive pathogen-associated molecular patterns lipoteichoic acid and peptidoglycan (13) or (ii) inhibiting the intracellular signaling cascade initiated upon TLR2 engagement by both cell wall components. If the latter explanation is true, then EPS could be considered a bona fide beneficial immunomodulator.

In summary, the comparative analysis of β-glucan-producing and nonproducing strains performed in this work has provided insights into the debated issues of probiotic properties of EPS-producing LAB (3) and the role of EPS in the immunomodulation of macrophages (20).

Our results indicate that *P. parvulus* 2.6 should be able to tolerate human GI stress and thus could be metabolically active in the colon. This supports the detected changes in short-chain fatty acid formation in the cecum, distal colon, and feces of rats fed with fermented oat-based food elaborated with this bacterium (15).

The EPS produced and secreted by LAB seem to be implicated in cellular recognition and the formation of biofilms, e.g., the glucans and fructans of *Streptococcus mutans* play an important role in the adhesion of this bacterium to the tooth surface and in the formation of dental plaque (14), thus facilitating bacterial colonization and protection against hostile habitats. However, the involvement of these biopolymers in bacterial adhesion to the intestinal epithelium in vivo has not yet been validated (25). The results of Dols-Lafargue et al. (4) show the contribution of the 2-substituted (1,3)-β-D-glucan to biofilm formation by LAB, and our results strongly support the involvement of this EPS in adhesion to human epithelial cells.

There are several reports that indicate a host immune response to LAB in which the involvement of various surface components of these bacteria is demonstrated (10, 22, 28). It has been reported that the suppressive effect on activation of macrophages exerted by *Lactobacillus casei* strain Shirota is associated with its EPS content (34). It is also known that the (1,3)-β-D-glucans can promote antitumor and antimicrobial activity by activating macrophages, dendritic cells, or other leukocytes (1, 24). The immune responses to eukaryote-derived glucans [either linear or with (1,6) branches] and to prokaryotic linear curdlan, used for making functional foods (tofu), have been characterized, and the activities of these molecules have been correlated with their chemical structure, molecular weight, and conformation (2, 23). However, the immunomodulating properties of β-D-glucans with (1,2) branches have not been reported until now. Therefore, this is the first report that a 2-substituted (1,3)-β-D-glucan affects the activation of human macrophages. Further experiments are in progress to characterize the influence of this β-D-glucan and of *P. parvulus* 2.6 on the immune response.

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