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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

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ABSTRACT

Exopolysaccharide production by lactic acid bacteria is beneficial in the dairy and oat-based food industries and is used to improve the texture of the fermented products. However, β-D-glucan–producing bacteria are considered spoilage microorganisms in alcoholic beverages because their secreted exopolysaccharides alter the viscosity of cider, wine, and beer, rendering them unpalatable. The plasmidic glycosyltransferase (*gtf*) gene of the *Pediococcus parvulus* 2.6 strain isolated from ropy cider has been cloned and sequenced, and its GTF product was functionally expressed in *Streptococcus pneumoniae*. The GTF protein, which has glycosyltransferase activity, belongs to the COG1215 membrane-bound glycosyltransferase family, and agglutination tests revealed that the enzyme enables *S. pneumoniae* to synthesize β-D-glucan. PCR amplification and Southern blot hybridization showed that the *gtf* gene is also present at different genomic locations in the β-D-glucan producers *Lactobacillus diolivorans* G77 and *Oenococcus oeni* I4 strains, also isolated from ropy cider. A PCR assay has been developed for the detection of exopolysaccharide-producing bacteria. Forward and reverse primers, included respectively in the coding sequences of the putative glycosyltransferase domain and the fifth *trans*-membrane segment of the GTF, were designed. Analysis of 76 ropy and nonropy lactic acid bacteria validated the method for specific detection of β-D-glucan homopolysaccharide producer *Pediococcus, Lactobacillus,* and *Oenococcus* strains. The limit of the assay in cider was 3×10^2 CFU/ml. This molecular method can be useful for the detection of ropy bacteria in cider before spoilage occurs, as well as for isolation of new exopolysaccharide-producing strains of industrial interest.

Lactic acid bacteria (LAB) produce a wide variety of exopolysaccharides (EPSs) located outside of the cell wall of different composition, molecular mass, and structure. These secreted sugar polymers are natural viscosifiers and texture enhancers of interest to the food industry but are deleterious to the organoleptic properties of fermented alcoholic beverages. In cider *(9),* wine *(16),* and beer *(26),* EPS-producing LAB are responsible for an alteration, called ''ropiness'' or ''oiliness,'' characterized by a viscous, thick texture and oily feel, which although not appreciably altering the taste, renders the products unpleasant to the palate. Ropy beverages are sometimes encountered either during elaboration or after bottling and cannot be sold, resulting in considerable financial loss.

EPSs from LAB are hetero- or homopolysaccharides. The heteropolysaccharides are composed of a repeating unit that contains two or more different monosaccharides, and the proteins involved in their synthesis and secretion are encoded by large operons, including more than 10 genes *(15).* In the homopolysaccharides, the repeating unit is composed of one type of monosaccharide. On the basis of their structures, they are classified as α-D-glucan, β-D-glucan, β-D-fructan, and others, such as polygalactan *(23).* It has been reported that β-D-glucan from several bacteria and fungi, in addition to being a biothickener, possesses antithrombotic, antitumoral, or immunomodulatory activity *(25)* and that oat β-D-glucan reduces serum cholesterol levels *(29).* Consequently, the influence of the β-D-glucan–producing LAB strains isolated from spoiled cider have been investigated for their use in the elaboration of functional oat-based products *(20),* as well as the physiological conditions to improve EPS production by the ropy *Pediococcus parvulus* 2.6 strain *(6).* (This strain was formerly called *Pediococcus damnosus* 2.6; see ''Materials and Methods: Identification of LAB.'') However, despite these advances, little is known about the molecular basis of β-D-glucan production by LAB. It has been established that *Pediococcus (8, 16)* and *Lactobacillus (7)* isolated from spoiled wine and cider produce an identical 2-substituted (1→3)-β-D-glucan. In *P. parvulus* 2.6 and *P. damnosus* IOEB8801 strains isolated from cider *(9)* and wine *(19),*

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respectively, the production seems to be linked to the presence of plasmids pPP2 (35 kb) and pF8801 (5.5 kb), respectively. PCR detection of β-D-glucan–producing bacteria has been approached with the use of primers, which render amplicons of the wine *P. damnosus* IOEB8801 5.5 kb plasmid. These amplicons were either in a region of the *mob* gene involved in plasmid transfer *(27)* or a region of the *dps* gene, which encodes a putative glucan synthase *(28).* The first method proved to be specific for the detection of ropy *P. damnosus* strains only, and the second method expanded the spectrum to the detection of one *Oenococcus oeni* ropy strain.

In this article, we report the cloning and molecular characterization of the *P. parvulus* 2.6 glycosyltransferase (*gtf*) gene, carried by the 35-kb pPP2 plasmid, which encodes a β-D-glucan synthase. From this characterization, we designed PCR primers, which permitted specific identification of β-D-glucan–producing strains of *Pediococcus, Lactobacillus,* and *Oenococcus.*

MATERIALS AND METHODS

Bacterial strains, isolation, and growth. *Streptococcus pneumoniae* R6 uncapsulated strain *(12)* was grown in casein hydrolysate–based AGCH medium *(14)* supplemented with 0.8% sucrose and 0.5% yeast extract for cloning of *gtf* or Tood-Hewitt medium (Difco, Becton Dickinson, Sparks, Md.) supplemented with 0.5% yeast extract, 0.1% sucrose, and 0.8% maltose for heterologous expression of the *gtf* gene. *Escherichia coli* DH5α (*supE44* Δ*lacU169* [ϕ80 *lacZM15*] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was grown in Luria-Bertani medium *(21)* and used as a host for cloning experiments. Table 1 shows the LAB strains used in this study. LABs from the collection of the Universidad del País Vasco (CUPV) were isolated from Basque Country ciders by previously published procedures *(5).* After incubation, the roping ability of the colonies was based on visual observation by touching them with an inoculation loop and assessing the occurrence of long ropy filaments. Moreover, ropy character was tested in liquid medium by detecting an increase in the viscosity. *Lactobacillus, Pediococcus,* and *Leuconostoc* strains were routinely grown at 28°C in deMan Rogosa Sharpe (MRS) medium (Difco, Becton Dickinson). *O. oeni* strains were grown in Leuconostoc oenos medium *(2)* at 28°C. *Streptococcus thermophilus* was grown at 37°C in MRS medium. The strains were stored at −80°C in 20% (vol/vol) glycerol.

Identification of LAB. The LAB strains isolated from cider were identified by biochemical tests, 16S rRNA sequence analysis, and a PCR method with species-specific primers.

The homo- or heterofermentative characteristic of the strains was tested as described by Dueñas et al. *(4).* Carbohydrate utilization was determined with the API 50CH system (API-bioMérieux, Marcy l'Etoile, France). API galleries were incubated for up to 7 days at 28°C.

For 16S rRNA sequence analysis of *Lactobacillus, Pediococcus,* and *Oenococcus* strains, a fragment of the 16S rDNA (corresponding to the positions 9 to 539 in the *Escherichia coli* numbering system) was amplified with primers PA (5′-AGAGTTTGATCCTGGCTCAG-3′) and UP1-R (5′-TACCG CGGCTGCTGGCAC-3′). Each 50-μl PCR reaction was carried out with 1.25 U of BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany) and contained 1 μM of each primer, 0.75 mM of $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 25 μM of each dNTP, and 5 μl of DNA template. Cycling conditions were 1× (95°C, 5 min); 35× (95°C, 1 min; 50°C, 1 min; 72°C, 30 s), and 1× (72°C, 10 min). The PCR products were separated on a 0.8% (wt/vol) agarose gel, and the amplicons were purified with the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA sequences of the amplicons of approximately 530 bp were determined and identified by BLAST searching of the GenBank DNA database. This analysis permitted classification of the new LAB strains isolated from cider shown in Table 1 and revealed that the 16S rRNA of the CUPV *P. damnosus* 2.6 and *Lactobacillus* spp. G77 strains, previously classified by biochemical methods, have a homology of 99 and 97%, respectively, with the 16S rRNA of *P. parvulus* and *Lactobacillus diolivorans.* Accordingly, the strains have been renamed in this work as *P. parvulus* 2.6 and *L. diolivorans* G77.

To confirm identity of *O. oeni* strains, a PCR amplification with species-specific primers was performed by the method described by Zapparoli et al. *(30).*

Purification of *P. parvulus* total plasmid DNA. Bacterial cultures were grown to an absorbance at 660 nm (A_{660}) of 1.5, and 100 ml of each culture was sedimented by centrifugation at 13,603 × *g* for 10 min at 4°C. The cells were lysed, and RNA was hydrolyzed by suspension in 4 ml of solution 1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, and 30 mg/ml lysozyme) plus 0.5 mg/ml RNase A and incubation for 30 min at 37°C. Cell debris and chromosomal DNA were removed from the extracts by sequential treatments for 5 min at 21°C with 4 ml of solution 2 (200 mM NaOH and 1% sodium dodecyl sulfate) and with 4 ml of 5 M potassium acetate pH 5.5, followed by centrifugation at 13,603 × *g* for 15 min at 21°C. The plasmidic DNA present in the supernatants was precipitated, concentrated by addition of 8.7 ml of isopropanol, centrifugation at 13,603 × *g* for 15 min at 4°C, and resuspension in 4.3 ml of 10 mM Tris, 1 mM EDTA (TE) buffer. The DNA preparation was deproteinated by treatment with 2.7 ml of 7.5 M ammonium acetate and 4.7 ml of phenol for 5 min at 21°C and centrifugation at 13,603 × *g* for 5 min at 21°C. The aqueous phase containing DNA was concentrated by precipitation with ethanol at 68% for 30 min at −20°C and centrifuged at 13,603 × *g* for 15 min at −10°C. The precipitated DNA was washed with 1 ml of 70% ethanol, sedimented by centrifugation at 13,603 × *g* for 15 min at −10°C, and resuspended in 200 μl of TE buffer.

Purification of LAB total genomic DNA. Bacterial cultures were grown to an A_{660} of 1.0, and 12 ml of each culture was sedimented by centrifugation at 13,603 × *g* for 10 min at 4°C. The cells were lysed by suspension in 1 ml of solution 1, incubated for 30 min at 37°C, and treated with 2.5% sodium dodecyl sulfate for 1 min at 37°C. The extracts were deproteinated by treatment with an equal volume of a mixture of phenol, chloroform, and isoamyl alcohol (50:48:2, vol/vol/vol) for 5 min at 21°C and centrifugation at 13,603 × *g* for 5 min at 21°C. The aqueous phase containing DNA was concentrated by precipitation with ethanol at 68% and 0.3 M sodium acetate pH 6.0 for 30 min at −20°C and centrifugation at 13,603 × *g* for 15 min at −10°C. The precipitated DNA was washed with 1 ml of 70% ethanol, sedimented by centrifugation at 13,603 × *g* for 15 min at −10°C, and resuspended in 200 μl of TE buffer.

Isolation of cider microbiota DNA. Cells from 1-ml cider samples were sedimented by centrifugation at 13,000 × *g* for 5 min and washed twice by resuspension in 1 ml of 0.9% NaCl and centrifugation at 13,603 × *g* for 5 min. Cells were resuspended in 200 μl of Instagene-Matrix resin (Bio-Rad, Hercules, Calif.),

incubated at 56°C for 30 min, and centrifuged at 11,000 × *g* for 5 min. The supernatants containing the DNA were mixed with 5 mg of polyvinylpyrrolidone, vortexed, and centrifuged at 13,000 × *g* for 10 min to remove inhibitors of DNA polymerases present in cider by sedimentation. The supernatants were kept frozen at −20°C before use in PCR reactions.

Cloning of the *gtf* region of *P. parvulus* 2.6. Plasmidic total DNA preparation of *P. parvulus* 2.6 was used as substrate in the following procedures. Cloning of the 5′ region of the *gtf* gene was performed by PCR amplification with use of the degenerated primers GTFDF (5′-TAYGAYAAYACNCARGARGT-3′) and GTFDR (5′-ACRAARTARTCRTARTCRTG-3′), where Y = T or C; W = A or T; R = A or G; and N = A, C, G, or T. These primers were designed with the use of the conserved amino acid sequence of the putative Dps glucan synthase of *P. damnosus* IOEB8801 *(27)*. The 1.8-kb amplicon was inserted into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, Calif.), and the recombinant plasmid pTGTF1 was established in *E. coli* DH5α. The 3′ and proximal downstream regions of *gtf* were cloned with use of the same vector and host as above, after reverse PCR amplification performed with the primers TGTFF (5′-ACGCCCTGCGTGT TATCATA-3′) and TGTFR (5′-TGTGTAATGGCACTCACGAC-3′) inferred from the DNA sequence of the pTGTF1 insert. In addition, shotgun cloning of digested total plasmid DNA permitted determination of the DNA sequence of the *gtf* region depicted in Figure 1A.

Construction of pGTF plasmid. The plasmidic DNA preparation of *P. parvulus* 2.6 was used for PCR amplification with primers *Xba*IF (5′-TCTAGAAATTAAAGGAATGTGTAA-3′) and *Xba*IR (5′-TCTAGATTAATCATTCCAATCAACTG-3′), with the *Xba*I restriction sites underlined, to obtain a 1,743-bp amplicon containing the *gtf* gene and its ribosomal binding site. The PCR product was then digested with *Xba*I and cloned into the unique *Xba*I site of the pLS1RGFP expression vector, which contains the green fluorescent protein gene cloned under the control of the pneumococcal P_M promoter *(24)*. The resulting plasmid, pPL100 carrying the P_M-*gtf-gfp* transcriptional fusion, was propagated in *S. pneumoniae* strain R61. Transformants were selected for erythromycin resistance, and the correct nucleotide sequence of the chromosomal insert of pGTF was confirmed by DNA sequencing.

DNA sequencing. Both DNA strands of amplicons and recombinant plasmid inserts were sequenced with the automated DNA sequencing instrument ABI PRISM 3730 (Applied Biosystems, Foster City, Calif.), at the Sequencing Service of the Centro de Investigaciones Biológicas.

PCR detection of the *gtf* region. For detection of the *gtf* gene, primers GTFF (5′-CGGTAATGAAGCGTTTCCTG-3′) and GTFR (5′-GCTAGTACGGTAGACTTG-3′) were used for amplification. Each 50-μl PCR reaction was carried out with 1.25 U of BIOTAQ DNA polymerase (Bioline) and contained 0.2 μM of each primer, 250 μM of each dNTP, 3.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl, and 1 μl of total genomic DNA or 5 μl of cider microbiota DNA as template. Cycling conditions were 1× (95 °C, 5 min); 30× (95°C, 1 min; 55°C, 1 min; 72°C, 30 s), and 1× (72°C, 10 min).

For amplification of the *gtf* gene upstream regions, we used the primers TRA1 (5′-CAACAAGCCAAGGACGACGACCA-3′), MOB1 (5′-TCTCATCAAGATGAACAATTGC-3′), and GTF1 (5′-ACGCCCTGCGTGTTATCATA-3′), whose sequences are included, respectively, in the *traA* gene of *P. parvulus* 2.6, the *mob* gene of *P. damnosus* IOEB18801 *(10)*, and in the *gtf* gene of *P. parvulus* 2.6. Each 50-μl PCR reaction was carried out with 2 U of Dnazyme DNA polymerase (Finnzymes, Espoo, Finland) and contained 0.2 μM of each primer, 250 μM of each dNTP, 3.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl, and 1 μl of total genomic DNA as template. Cycling conditions were 1× (95°C, 2 min); 30× (95°C, 1 min; 50°C, 1 min; 72°C, 1.5 m), and 1× (72°C, 10 min).

Southern blot hybridization. Total genomic (4 μg) or plasmidic (1.5 μg) DNA preparations fractionated in a 0.6% agarose gel were transferred to a 0.45-μm nylon membrane (Biodyne A, Pall Corporation, Ann Arbor, Mich.) and hybridized with a probe at 65°C. Probe labeling and detection procedures were performed with the NEBlot Phototope Kit (BioLabs, Ipswich, Mass.) and Phototope Stars Detection Kit (Biolabs). The substrate for probe labeling was an amplicon of 598 bp synthesized with primers GTFSF (5′-TTGCCAGAACTAGAGAAAGTACGCA-3′) and GTFSR (5′-ACTTCCTATTTTAGCTAAAAAGCAA-3′) and plasmidic *P. parvulus* 2.6 DNA template.

Immunological analysis. Agglutination tests were performed with *S. pneumoniae* type 37–specific antisera. *S. pneumoniae* R61[pLS1RGFP] and R61[pGTF] were grown in Tood-Hewitt medium supplemented with 0.5% yeast extract, 0.1% sucrose, and 0.8% maltose to an A_{650} of 0.4. Each culture (1 ml) was sedimented by centrifugation, and cells were resuspended in 100 μl of PBS pH 8.0 (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl). Each culture (10 μl) was mixed with 10 μl of *S. pneumoniae* type 37–specific antisera (Statens Seruminstitut, Copenhagen, Denmark) and incubated for 2 h at 4°C. The preparations were analyzed by phase contrast and fluorescent microscopy with a Zeiss Axioplan (Universal microscope, Stuttgart, Germany) bearing a standard fluorescein isothiocyanate set of D480/30 excitation and TBP 460/530/610 emission filters for total bacterial cells and cells expressing the green fluorescent protein, respectively.

Membrane preparation and detection of glycosyltransferase activity. *S. pneumoniae* R61[pLS1RGFP] and R61[pGTF] were grown in 1 liter of Tood-Hewitt medium supplemented with 0.5% yeast extract, 0.1% sucrose, and 0.8% maltose to an A_{650} of 1.1. Membrane isolation and glycosyltransferase assays were preformed as described by Llull et al. *(17)*. Briefly, cells from 1-liter cultures were resuspended in 5 ml of solution 3 (70 mM Tris-HCl pH 7.0, 9 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.2 M phenylmethylsulfonyl fluoride) after sedimentation and disrupted by two passages through a French Press (Aminco, New York). After removal of cell debris by centrifugation at 800 × *g* for 10 min at 4°C, membranes were sedimented by centrifugation at 120,000 × *g* for 30 min at 4°C, resuspended in 2 ml of solution 3, and stored at −70°C. Protein content of the membrane extracts was determined with the Bradford BCA Protein Assay (Pierce, Rockford, Ill.). Each 100-μl glycosyltransferase reaction contained membrane extracts at concentrations ranging from 0.5 to 5.0 mg/ml of proteins, 30 μM UDP-[^{14}C] glucose (specific activity 333 mCi/mmol), solution 3, and 50 mM NaCl. Reactions were performed at 30°C for 15 min and stopped by the addition of sodium dodecyl sulfate (final concentration 0.5%). After addition of bovine serum albumen (final concentration 0.4%) and 1 ml of 10% trichloroacetic acid, samples were incubated for 30 min at 0°C, passed through Whatman GF/A filters, and washed with 15 ml of 10% trichloroacetic acid. Filters were dried at 65°C for 20 min and counted in a scintillation counter (LKB Wallack, Bromma, Sweden). One unit of glycosyltransferase activity is expressed as the enzyme concentration that catalyzes the incorporation into a macromolec-

TABLE 1. *Detection of* gtf *gene in EPS-producing LAB by PCR*

Bacterium	Source[a]	No. of strains analyzed	EPS phenotype/type of EPS[b]	PCR detection of *gtf*
Pediococcus parvulus 2.6	CUPV (Basque Country ropy cider *(9)*)	1	+/β-D-glucan *(8)*	+
P. parvulus	CUPV (Basque Country ropy cider)	14	+	+
P. parvulus 4794	CECT	1	−	−
P. damnosus 4693	CECT	1	+	+
P. damnosus 4694	CECT	1	−	−
P. pentosaceus 4695^T	CECT	1	−	−
P. pentosaceus 4692	CECT	1	−	−
Lactobacillus diolivorans G77	CUPV (Basque Country ropy cider *(4)*)	1	+/β-D-glucan *(7)*	+
L. diolivorans	CUPV (Basque Country ropy cider)	15	+	+
L. suebicus	CUPV (Basque Country ropy cider)	2	+	+
L. collinoides	CUPV (Basque Country ropy cider)	3	+	+
L. collinoides	CUPV (Basque Country nonropy cider)	2	−	−
L. collinoides 922^T	CECT	1	−	−
L. plantarum	CUPV (Basque Country nonropy cider)	2	−	−
L. plantarum 223	CECT	1	−	−
L. brevis 216	CECT	1	−	−
L. mali 4149	CECT	1	−	−
L. hilgardii 4786^T	CECT	1	−	−
L. delbrueckii subsp. *bulgaricus* 702772	NCIMB	1	+/Heteropolysaccharide *(11)*	−
L. helveticus 700766	NCIMB	1	+/Heteropolysaccharide *(3)*	−
Oenococcus oeni I4	CUPV (Basque Country ropy cider *(13)*)	1	+/β-D-glucan *(13)*	+
O. oeni	CUPV (Basque Country ropy cider)	3	−	−
O. oeni	CUPV (Basque Country nonropy cider)	15	−	−
O. oeni 218	CECT	1	−	−
Leuconostoc mesenteroides B742	NRRL	1	+/α-D-glucan *(23)*	−
L. mesenteroides subsp. *mesenteroides* 394	CECT	1	+/α-D-glucan *(23)*	−
L. mesenteroides subsp. *cremoris* 873	CECT	1	−	−
Streptococcus thermophilus 700859	NCIMB	1	+/Heteropolysaccharide *(3)*	−

[a] Institutional names: CUPV, Colección de la Universidad del País Vasco (Spain); CECT, Colección Española de Cultivos Tipo (Universidad de Valencia, Burjassot, Spain); NCIMB, National Collection of Industrial and Marine Bacteria (Aberdeen Scotland, UK); NRRL, Agricultural Research Service (NRRL) Culture Collection (Peoria, Illinois, USA).

[b] The EPS phenotype (+ or −) was assigned by visual examination. The EPS type had been previously established by determination of the EPS structures in the corresponding references.

ular product of 1 pmol of glucose per mg of total proteins per min.

RESULTS

Characterization of the *gtf* gene and GTF protein of *P. parvulus* 2.6. The DNA sequence of the *dps* gene carried by the *P. damnosus* pF8801 plasmid has not been published. However, the partial amino acid sequence of the putative glucan synthase Dps protein, homologous to glycosyltransferases, is available *(27)*. This sequence allowed us to clone the *P. parvulus* 2.6 *gtf* gene and its flanking regions after PCR amplification of a 5′ fragment of *gtf* with degenerated oligonucleotides and further reverse PCR and shotgun cloning (see details in ''Materials and Methods''). Determination of the DNA sequence of the 4,535-nt cloned *gtf* region revealed the existence of three open reading frames designated *traA*, *gtf*, and *tnp* (Fig. 1A). Comparison of these DNA sequences with the nucleotide National Center for Biotechnology Information (NCBI) database revealed a cassette structure for this region. The 1,700-nt *gtf* (nucleotides 1,284 to 2,983) has no significant homology with other sequenced genes, However, a 168-nt sequence (nucleotides 842 to 1,009) located between *traA* and *gtf* showed 91% identity with a region of the *L. plantarum* pWCFS103 plasmid, and a 1,136-nt sequence (nucleotides 3,400 to 4,532), including *tnp* and positioned downstream of *gtf*, showed an identity of 91% with a genomic DNA region of *L. rhamnosus* RW-9595M, which is located upstream of an EPS biosynthetic operon (GeneBank AF323526). A search of the Swissprot database with the amino acid sequence of the inferred gene products revealed that *traA* encodes a relaxase, presumably involved in conjugation, with high homology (92 to 59%) to its counterparts of *Lactobacillus* (pSF118-20, pWCFS1, and pSF118-48) and *Lactococcus lactis* (pMRC01) plasmids and that TraA has no homology with the Mob protein encoded by the pF8801 plasmid of *P. damnosus* IOEB18801 (*(10)*; Fig. 1A). The *tpn* gene encodes a putative transposase belonging to the IS30 family, which is widespread among *Lactobacillus* species.

The *gtf* gene product (GTF) belongs to the COG1215 membrane-bound glycosyltransferase family. Topology pre-

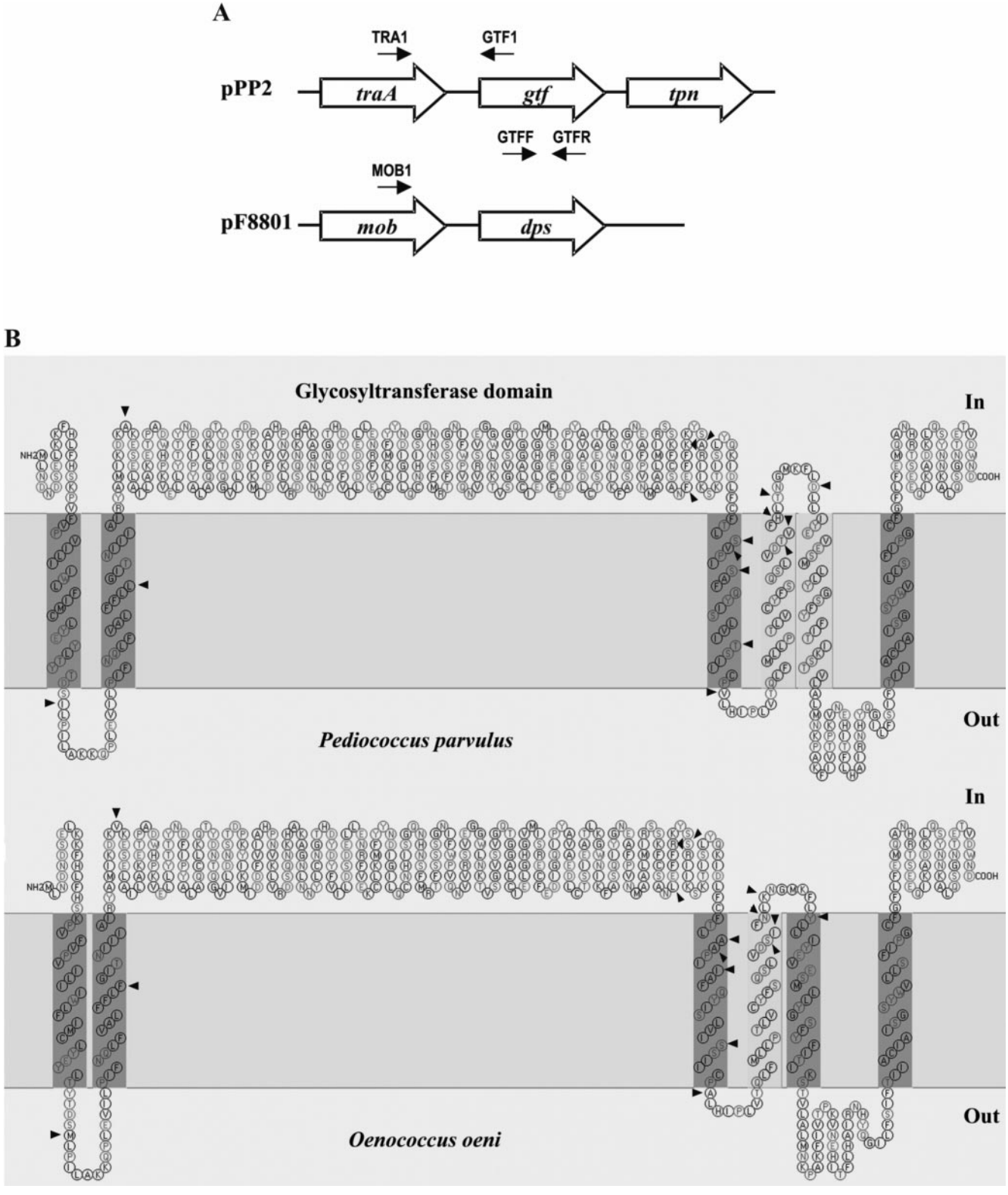

FIGURE 1. *The* gtf *region and the GTF protein of* P. parvulus *2.6. (A) Representation of the regions including the glucan synthase genes of plasmids pPP2 and pF8801 from* P. parvulus *2.6 (inferred from DNA sequencing) and* P. damnosus *IOEB8801 (inferred from Walling et al. (27)). Location of the primers used in the experiments depicted in Figures 4 and 5 are indicated. (B) Secondary structure for GTF glycosyltransferases of* P. parvulus *2.6 and* L. diolivorans *G77 (upper part) and* O. oeni *I4 (lower part) predicted by the SOSUI program (22). Amino acids not conserved in both GTFs are indicated by arrowheads.*

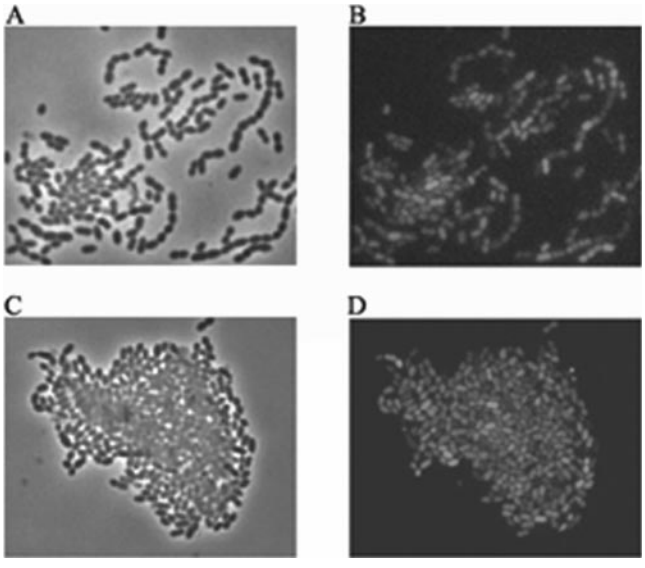

FIGURE 2. *Detection of GTF and green fluorescent protein functional expression from P_M promoter in* S. pneumoniae. *Pictures of R61[pLS1RGFP] (A and B) and R61[pGTF] (C and D) cultures after agglutination tests and detection by contrast phase (A and C) and fluorescent (B and D) microscopy are depicted.*

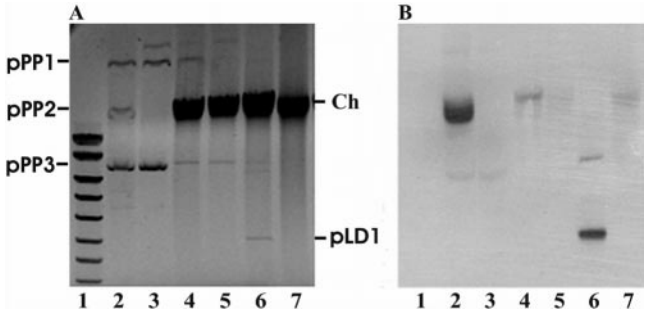

FIGURE 3. *Detection of* gtf *genomic location in LAB by Southern blot hybridization. (A) Agarose gel stained with ethidium bromide. (B) Autoradiogram of the hybridized membrane. Lane 1, molecular mass standard (Smartladder, Eurogentec, Seraing, Belgium); 2, total plasmidic DNA of* P. parvulus *2.6; 3, total plasmidic DNA of* P. parvulus *2.6NR; 4, total genomic DNA of* P. parvulus *2.6; 5, total genomic DNA of* P. parvulus *2.6NR; 6, total genomic DNA of* L. diolivorans *G77; 7, total genomic DNA of* O. oeni *I4. The position of chromosomal DNA (CR) and of the circular covalently closed forms of plasmids pPP1, pPP2, and pPP3 of* P. parvulus *2.6 and pLD1 of* L. diolivorans *G77 are indicated.*

diction of the GTF protein (Fig. 1B) with the S0SUI program *(22)* indicates that it is a membrane-bound protein. The GTF has a significant identity (33%) only with the Tts glycosyltransferase of *S. pneumoniae* serotype 37 *(18).* This enzyme catalyzes the biosynthesis and secretion of the capsule of this microorganism *(17),* which is a β-D-glucan similar to the EPSs synthesized by *P. damnosus* and *P. parvulus.* Antibodies against serotype 37 are able to agglutinate *P. damnosus* IOEB8801 cells *(28)* and other gram-positive bacteria overexpressing Tts *(17).*

On these bases and to test the function of GTF, the *gtf* gene was located under the control of the P_M promoter in a transcriptional fusion of P_M-*gtf-gfp* by cloning in the pLS1RGFP expression vector (see details in ''Materials and Methods''). The recombinant plasmid pGTF was established in the *S. pneumoniae* R6 uncapsulated strain, and expression of *gtf* was analyzed by immunoprecipitation with antibodies against serotype 37 and visualization under the microscope (Fig. 2). Comparison of the images obtained with phase contrast (Fig. 2A and 2C) and fluorescent (Fig. 2B and 2D) microscopy showed that all cells carrying either the pGTF (Fig. 2D) or the vector (Fig. 2B) plasmids were fluorescent because of the green fluorescent protein transcribed from the P_M promoter. However, only R6[pGTF] cells (Fig. 2C and 2D) showed agglutination with the serotype 37 antibody. This result indicates that expression of GTF confers to *S. pneumoniae* the ability to synthesize and secrete the EPSs.

To confirm GTF glycosyltransferase activity, membranes of R6[pGTF] and R6[pLS1RGFP] were prepared and tested with UDP-[^{14}C]-glucose substrate. The levels of glycosyltransferase activity detected in the GTF overexpressor and control strain were 615 ± 193 and 244 ± 42 U/mg of total protein, respectively. The high background activity observed in R6[pLS1RGFP] could be from the products of *cpoA* and *epsG* chromosomal genes of *S. pneumoniae,* which have been proposed to be glycosyltransferases by homology in the KEGG database *(1).* However, the 2.5-fold increase in activity detected in R6[pGTF] membranes seems to be from GTF expression because no significant alteration of the doubling time was observed (35 min for the overproducer strain compared with 30 min for the control strain), and in both strains, the P_M promoter was active (results not shown).

Genomic location of *gtf* in LAB. The above results strongly indicated that GTF is indeed a glucan synthase and that strains synthesizing β-D-glucan should carry the *gtf* gene. To test this hypothesis and determine the location of *gtf,* total genomic DNA from *L. diolivorans* G77 and *O. oeni* I4, which synthesize the same EPS as *P. parvulus* 2.6 *(8, 13),* were tested by Southern blot hybridization with a probe containing an internal fragment of the *gtf* gene (Fig. 3). In *Lactobacillus* and *Oenococcus* samples, hybridization signals (Fig. 3B) were observed at the position of the 5.5-kb plasmid designated pLD1 and of chromosomal or high-molecular mass plasmidic DNA, respectively (Fig. 3A). *P. parvulus* 2.6 carries three plasmid designated pPP1, pPP2, and pPP3 (Fig. 3A); as expected, the probe hybridized (Fig. 3B) with the 35-kb pPP2 plasmid, and no hybridization was observed with plasmidic DNA of the isogenic strain *P. parvulus* 2.6NR cured of pPP2, which is unable to synthesize the β-D-glucan *(9).*

To confirm a different location of *gtf* in the strains tested, PCR amplifications of the *gtf* upstream regions were performed (Fig. 4B and 4C). As a control, amplification of an internal region of the gene was also performed (Fig. 4A). As expected, the internal amplicon of *gtf* was detected by use of the genomic DNA of the three LAB strains (Fig. 4A). However, amplification performed with primers TRA1 and GTF1, located in the *traA* and in the *gtf* genes of *P. parvulus* 2.6, respectively, only yielded the expected amplicon with DNA from *Pediococcus* (Fig. 4C). Moreover,

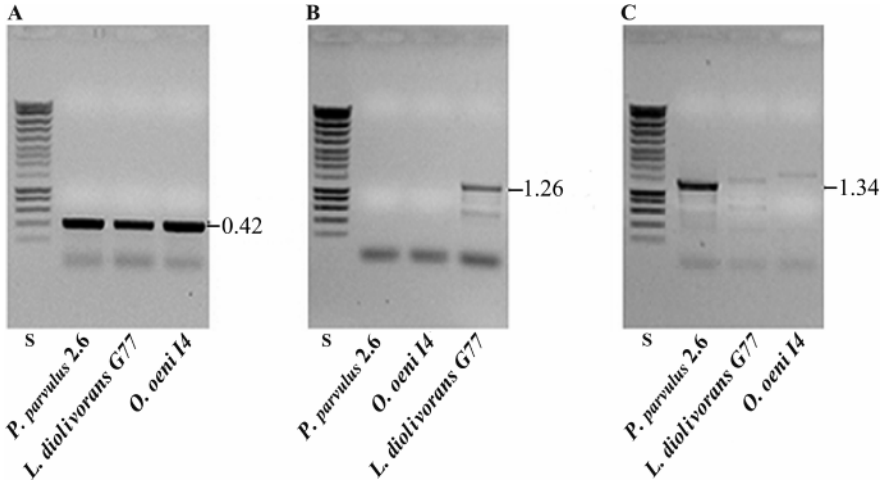

FIGURE 4. *PCR detection of the* gtf *gene and upstream regions in the genome of LAB. Agarose gel analysis of PCR reactions performed with total DNA preparation of the LAB indicated and the primers GTFF and GTFR (A), Mob1 and GTF1 (B), or TRA1 and GTF1 (C). S, molecular mass standard (Smartladder, Eurogentec). The length of the amplicons (kb) is indicated.*

only amplification was obtained with the *Lactobacillus* substrate when the MOB1 (located in the *mob* gene of the 5.5-kb pF8801 plasmid of *P. damnosus* IOEB18801 (*(10)*; GeneBank AF196967) and the GTF1 primers were used (Fig. 4B). These results demonstrated a different genomic location for *gtf* in the three LAB strains tested. Furthermore, determination of the nucleotide sequence of the *L. diolivorans* G77 (GeneBank AY999684) and *O. oeni* I4 (GeneBank AY999685) *gft* genes after PCR amplification revealed that they possess a 100 and 98.8% identity, respectively, with their counterparts in *P. parvulus.*

PCR method for detection of β-D-glucan producer strains. The above results showed that the *gtf* gene is highly conserved in 1→3-β-D-glucan–producing LAB strains.

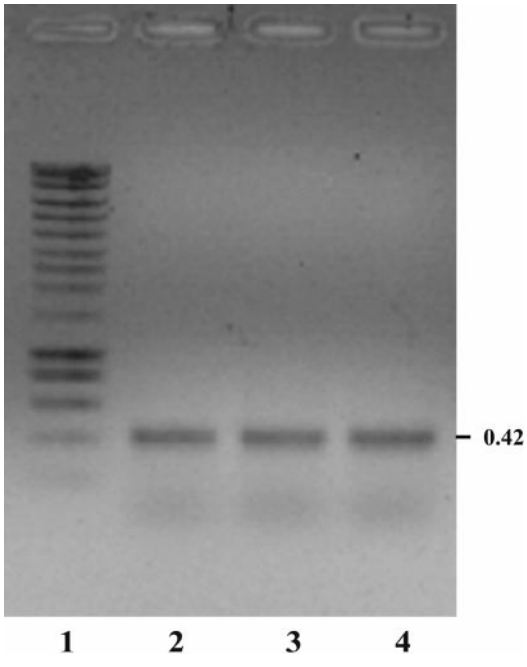

FIGURE 5. *PCR detection of* gtf *carrier LAB in cider. Agarose gel analysis of PCR reactions performed with primers GTFF and GTFR and with DNA isolated from cider inoculated with* P. parvulus *2.6 (lane 2) and from two batches of ropy cider (lanes 3 and 4). Lane 1, molecular mass standard (Smartladder, Eurogentec). The length of the amplicons (kb) is indicated.*

Therefore, this gene could be an appropriate target for PCR detection of β-D-glucan–producing LAB. To investigate this assumption, several primers based on *gtf* were tested on a panel of strains comprising 76 ropy and nonropy isolates from Basque Country ciders, as well as reference strains (Table 1) for PCR detection of *gtf* (data not shown). The highest specificity was obtained with the pair GTFF (positions 2,264 to 2,283, forward) and GTFR (complementary to the sequence spanning positions 2,663 to 2,680, reverse). (Coordinates are according to the *gtf* region sequence deposited under GeneBank AY551933.) These primers are located in the coding sequences of the putative carboxyl glycosyltransferase domain and the fifth TMS of GTF, respectively, and are predicted to give an amplicon of 417 bp according to the *P. parvulus* 2.6 *gtf* sequence (Fig. 1B). The expected amplicon was not detected with DNA from α-glucan homopolysaccharide- and heteropolysaccharide-producing strains or from nonropy strains. However, the presence of *gtf* was detected in all the ropy strains isolated from cider, including 15 *P. parvulus*, 16 *L. diolivorans*, 2 *Lactobacillus suebicus*, 3 *Lactobacillus collinoides*, and 1 *O. oeni* strains. Moreover, analysis of type collection strains expanded the spectrum of dissemination of *gtf* to the *P. damnosus* species. These results confirmed the specificity of the GTFF and GTFR primers for detection of *β*-D-glucan–producing strains.

Most of the *Lactobacillus* (21 of 25) and all *Pediococcus* (15 of 15) strains isolated from Basque Country ciders showed the ropy phenotype. However, among 20 *O. oeni* tested, only one strain showed the ropy phenotype.

The suitability of the primers GTFF and GTFR for PCR detection of β-D-glucan–producing strains in cider was analyzed (Fig. 5). Inoculation of cider with *P. parvulus* 2.6 and PCR tests performed with DNA isolated from serial dilutions of cider revealed that the limit of detection of this assay is 3×10^2 CFU/ml. In addition, two batches of spoiled cider were also tested, and the expected amplicon was detected (Fig. 5). These results reveal that in ropy cider, β-D-glucan producer strains indeed are present and can be detected by the presence of their *gtf* gene.

DISCUSSION

LAB producing EPSs have traditionally been considered spoilage bacteria because they are responsible for the

production of ropiness in cider, beer, and wine. In contrast, recent work on oat-based food elaborated with the β-D-glucan producers *L. diolivorans* G77 or *P. parvulus* 2.6 isolated from cider indicates their potential usefulness for elaboration of functional food *(20).* Considering the industrial interest in these organisms, this work aimed to develop a molecular method for the detection of β-D-glucan–producing bacteria. The gene *gtf* from *P. parvulus* 2.6 encoding the GTF glycosyltransferase, which catalyzes the synthesis of the homopolysaccharide β-D-glucan, has been cloned and sequenced, and GTF has been functionally expressed *in S. pneumoniae.* The *gtf* gene has no homology with any DNA sequence deposited in the databases. However, we have shown (Table 1) its wide occurrence among lactobacilli and pediococci, indicating its potential utility for the molecular detection of β-D-glucan–producing LABs in alcoholic beverages. The gene was also detected in one *O. oeni* strain. In this strain, the *gtf* gene seems to be chromosomally located, whereas in *P. parvulus* 2.6 and *L. diolivorans* G77 it is carried by plasmids. A small mobilizable plasmid encoding a putative glycosyltransferase has also been described in a ropy *P. damnosus* strain from wine *(10).* Thus, horizontal transfer mediated by plasmids could explain the wide distribution of the *gtf* gene among *Lactobacillus* and *Pediococcus* strains isolated from cider. These plasmids would not be able to replicate in *Oenococcus*; as a consequence, the incidence of the *gtf* gene in this bacterium is low. In addition, in *P. parvulus* 2.6, *gtf* is close to a putative transposase-coding gene, suggesting that transposition events also contribute to *gtf* dispersion among LABs.

Topology prediction of the identical GTF proteins of *P. parvulus* and *L. diolivorans* (Fig. 1B) indicates that a conserved cytosolic glycosyltransferase domain is flanked by two and four *trans*-membrane segments (TMSs). In the case of *O. oeni*, the 20 nucleotide changes detected in its *gtf* gene are curiously all clustered in the 5′ and 3′ regions of the gene and result in four silent mutations and 16 amino acid changes. Interestingly, 12 mutations are located in the predicted second (L73F), third (S381A, V382A, S385I, and T395S), and fourth (T428S, V429I, and H431N) TMSs of the *P. parvulus* GTF or in its first (I45M), second (V401A), and third (D440Y and T433K) loops interconnecting TMSs. The other four mutations are present in the proximal amino (A91V) and carboxyl (F351L, K355R, and A359S) regions of the GTF glycosyltransferase domain (see Fig. 1B). As a consequence of these mutations, the predicted secondary structure of the *O. oeni* GTF (Fig. 1B) shows alterations in the topology and amino acid composition for five of its six TMSs and in all the loops between TMSs. Therefore, these alterations could reflect evolutionary adaptive changes that permit an optimal anchoring of the GTF to the *Oenococcus* membrane.

Detection of glycosyltransferase activity in membranes isolated from an *S. pneumoniae* GTF-overproducing strain supports that GTF is a membrane-bound protein and confirms that it is indeed a glycosyltransferase. Moreover, the agglutination capacity conferred to *S. pneumoniae* by GTF overproduction strongly suggests that, like the pneumococcal Dps glycosyltransferase *(17),* the protein is responsible for synthesis and secretion of the β-D-glucan.

The *gtf*-based PCR method developed in this work allows the quick and specific detection of β-D-glucan–producing LABs of potential industrial interest. Furthermore, optimization of the method for alcoholic beverages will permit early detection of contamination, which in turn will allow processing decisions (i.e., sulfiting) to be made before the onset of ropiness. This offers a great advantage over the current situation, in which contamination of beverages by LABs is not detected until ropiness has appeared and the product has to be discarded at considerable financial loss.

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