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(71) Applicants:
• **Consejo Superior de Investigaciones Cientificas
28006 Madrid (ES)**
• **Universidad De Cantabria
39005 Santander (ES)**
• **UNIVERSIDAD DEL PAIS VASCO
48940 Leioa (Vizcaya) (ES)**

(72) Inventors:
• **Lopez Garcia, P.,
Centro de Investigaciones Biol.
28040 Madrid (ES)**

- **Werning, María, L.,
Centro Investigaciones Biol.
28040 Madrid (ES)**
- **Iratorza Iribas, Ana
E-48940 LEIOA (ES)**
- **Duenas Chasco, María Teresa
E-48940 LEIOA (ES)**
- **Ibarburu Lopez, Idoia
48940 LEIOA (ES)**
- **Navas Mendez, Jesús
39005 SANTANDER (ES)**

(74) Representative: **Ungria Lopez, Javier
Avda. Ramon y Cajal, 78
28043 Madrid (ES)**

(54) **GTF CELLS, VECTORS AND SEQUENCES AND APPLICATIONS THEREOF IN THE FOOD SECTOR**

(57) The invention relates to GTF micro-organisms, vectors and sequences which are formed by or comprise gene *gtf* de *P.damnosus* 2.6 which encodes a glycosyl-transferase that is located in the membrane and which enables the production of exopolysaccharides of the -glucan type by overexpression of said recombinant gene. The fact that only one gene is involved in the synthesis of the aforementioned enzyme facilitates the optimisation

of the expression conditions which enable the production of -glucans on an industrial scale. Said polymers, which can be used as additives in numerous foods, also have properties that are beneficial to human health. Moreover, said transformed GTF micro-organisms can be used in processes for the fermentation of foods, such as dairy products, alcoholic drinks and oats.

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Description

SECTOR OF THE ART

- 5 [0001] Biotechnology for the food sector. More specifically, fermentation of foods and drinks and in particular the obtaining of micro-organisms that produce exopolysaccharides (-glucans) and food additives.

STATE OF THE ART

- 10 [0002] Exopolysaccharides (EPSs) secreted by various bacteria play an important role as adherence factors in the interactions of bacteria with plants as signalling molecules or as protector agents towards situations of stress or infections by phages (van Kranenburg et al. (1999) Curr. Opin. Biotech. 10:498-504). EPSs are produced by both Gram-negative and Gram-positive bacteria. Some of these polymers possess unique properties that make them suitable for being used as stabilising agents, thickeners, gelling agents and emulsifiers. They are used as additives in the manufacture of numerous foods such as dairy derivatives (yoghurts, shakes and ice-creams), fruit juices and cereal-based preparations. Among the most representative EPSs produced by Gram-negative bacteria we can mention xanthan, produced by *Xanthomonas campestris* and used in cosmetics in rubber form as a thickener, stabiliser and suspension agent (Becker et al. (1998) Appl. Microbiol. Biotechnol. 50: 145-152). Also acetan, produced by *Acetobacter xylinum* is used for the production of substitutes for cream and vinegar (Sutherland (1998) Trends Biotechnol. 16: 41-46). Moreover, capsular polysaccharides produced by *Sphingomonas*, which possess viscous properties, are used as stabilising agents for foods (Sutherland (1998) Trends Biotechnol. 16: 41-46).

- 20 [0003] Gram-positive bacteria and specifically lactic acid bacteria (LAB) produce various EPSs of industrial interest. A clear example is dextrans produced by *Leuconostoc mesenteroides*, used in the synthesis of matrices employed in chromatography techniques for coating metal surfaces or for stabilising syrups. Moreover, some strains of LAB belonging to the genera *Streptococcus*, *Lactobacillus* and *Lactococcus* produce EPSs which are being used in situ for improving the texture of fermented products such as yoghurt and cheese (de Vos (1999) Cur. Opin. Biotechn. 10: 483-484). Nevertheless, the large scale production of EPSs from LAB is currently restricted owing to the low levels produced (40-800 mg l⁻¹ of culture) in comparison with *Xanthomonas campestris* which can produce from 10 to 25 g l⁻¹ or convert 60 to 70% of substrate sugar into xanthan by continuous culture.

- 30 [0004] Moreover, studies have been conducted which indicate that the EPSs produced by LAB possess beneficial effects for human health as stimulators of the immune system and it has therefore been proposed to use the producer strains in order to obtain functional foods (Jolly et al. (2002) Antonie van Leeuwenhoek 82: 367-374). Moreover, some of the EPSs are -glucans and there exists evidence that these homopolysaccharides contribute to reducing the levels of serum cholesterol (Behall et al. (1997) J. Am. College Nutr. 16: 64-51) Hence the interest in incorporating -glucans into foods and dietary products and the need to have efficient methods for their large scale production.

- 35 [0005] The structural characterisation of EPSs produced by strains isolated from cider *P. damnosus* 2.6 (Duenas et al. Carbohidr. Res. 303: 453-458), *Lactobacillus* sp. G77 (Dueñas et al. Carbohidr. Res. 307: 125-133) and *Oenococcus oeni* 14 (results not published) revealed that they all synthesised the same homopolysaccharide of the type -D-glucan. It has also been demonstrated that both *P. damnosus* 2.6 and *Lactobacillus* sp. G77 can grow and produce EPS during the production of fermented foods based on oats, improving their organoleptic qualities (Olof Mårtensson et al. (2002) Nutrition Research 22: 1461-1473).

- 40 [0006] A high proportion of the world adult population currently displays intolerance to lactose and a social awareness is being created around the allergic reactions produced by milk and soy. Oat is a cereal which possesses a high nutritional value owing to its content in proteins, vitamin E, unsaturated fatty acids and essential minerals. It also contains 4-6% of -glucans, which reduce the level of serum cholesterol in human beings. This effect has been noted in clinical studies on the ingestion of oat-based foods (see details at <http://www.adavena.com>) and in fact the FDA (Food and Drug Administration, USA) has authorised the labelling "product beneficial for health" in food preparations based on oat containing 0.75 g of soluble fibre (- glucan). The company Cereal Base (CEBA) (Lund, Sweden) specialises in the production of foods based on cereals as an alternative to dairy products. It has developed a technology known as Adavena which has permitted an oatmilk to be obtained with which products can be prepared as substitutes for yoghurts, dairy desserts, ice-creams, etc. fermented with LAB (Mårtensson et al., 2002). The nutritional and dietary properties of these preparations are improved if the content of -glucan in them is increased, by incorporating the producer bacteria or by the direct addition of these polymers.

- 50 [0007] Nevertheless, the genes or metabolic pathways involved in this production of -glucans in micro-organisms of potential usefulness in the food sector are not known.

DESCRIPTION

Brief description

[0008] An object of the present invention consists of a DNA sequence, hereinafter gtf DNA sequence of the present invention, coming from GRAS (Generally Recognised as Safe) lactic acid bacteria and encoding a protein with glycosyltransferase activity which permits the production of exopolysaccharides (EPSs) and which consists of one of the nucleotide sequences selected from between:

- a) the nucleotide sequence identified as SEQ ID N09 or a fragment thereof; and
- b) a nucleotide sequence analogous to the sequence defined in a).

[0009] The invention also refers to a GTF expression vector which comprises the GTF DNA sequence of the invention, hereinafter the GTF expression vector of the invention, and which permits the transformation of micro-organisms or cells and the subsequent obtaining of micro-organisms capable of expressing the GTF protein (SEQ ID NO6) and which permits the host to acquire the capacity, or improve the already existing capacity, to produce EPS.

[0010] Another object of the present invention is the use of the GTF DNA sequence or the GTF expression vector in a cell transformation process in order to obtain GTF cells.

[0011] A further object of the invention consists of a protein with glycosyltransferase activity which consists of one of the sequences of amino-acids selected from between:

- a) the amino acid sequence identified as SEQ ID N06 or a fragment thereof; and
- b) an amino acid sequence analogous to the sequence defined in a).

[0012] Another object of the invention consists of a cell, hereinafter the GTF cell of the present invention, which comprises a sequence of GTF DNA or a pGTF expression vector of the invention, capable of expressing in recombinant form the GTF protein (SEQ ID NO6) and which therefore acquires the capacity, or improves the already existing capacity, to produce EPS.

[0013] Another object of the present invention is the use of the GTF cells for producing EPSs by means of a procedure comprising:

- a) the growth of the GTF cell in a suitable medium and under conditions which permit expression of GTF expression vector or of GTF DNA sequence,
- b) the production of EPSs by the GTF cell of a), and
- c) purification of the EPSs of b)

Finally, a further object of the invention consists of the use of the GTF cells in fermentation processes for foods, among others and by way of illustration without limiting the scope of this invention, those belonging to the following group: dairy products, alcoholic drinks and oats.

Detailed description

[0014] The invention tackles the problem of providing new genetic and biological tools permitting the expression of new enzymes useful for the production of EPSs and the generation of micro-organisms of industrial value.

[0015] The solution proposed by this invention is based on the fact that the inventors have identified the gene gtf coding an enzyme with glycosyltransferase (GTF) activity, starting from genome material of *Pediococcus damnosus* 2.6. The recombinant expression of this gene in *Streptococcus pneumoniae* and in *Lactococcus lactis* demonstrated the glycosyltransferase activity of the enzyme encoded by that gene, along with the possibility of using that gene to successfully transform Gram-positive bacteria, including lactic acid bacteria, which acquire the capacity of its expression. The strains of lactic bacteria can be useful for obtaining EPSs for their later use as additives in the food sector and, moreover, their later genetic manipulation in order to confer GRAS quality on them which permits their use in the transformation of foods with new properties. The overproduction of GTF with glycosyltransferase activity could permit those polymers to be obtained on the large scale for their incorporation as additives in foods and dietary preparations which possess beneficial properties for human health.

[0016] The comparison of the sequence of that gtf gene with those deposited in databases showed the absence of significant homologies. Nevertheless, when comparing the sequence of the GTF protein product of the gtf gene it was observed that it is homologous with glycosyltransferases of COG1215 family. Moreover, the GTF protein presents a relatively high (34%) homology with the Tts protein of *Streptococcus pneumoniae*, a glycosyltransferase which is the only enzyme required for the biosynthesis and secretion of -D-glucan similar to that of *P. damnosus* (Llul et al. (1999)

J. Biol. Chem. 24: 21053-21061). This characteristic contrasts with the operon complexes (consisting of ten genes) which encode proteins required for synthesis and secretion of bacterial heteropolysaccharides and homopolysaccharides currently used at the industrial level (Jolly and Stingle (2001) Int. Dairy J. 11: 733-745) and represents a clear and important technical advantage. As a consequence, a protein with similar characteristics to Tts, but coming from a non-pathogenic micro-organism having food quality would have potential for the production of -D-glucans by genetic manipulation and would be of industrial interest.

[0017] The same research team has observed that, by means of the PCR characterisation of the gene *gtf* in different bacterial strains producing -D-glucan, of homo- or hetero-polysaccharides, the protein GTF is specifically implicated in the biosynthesis of homopolysaccharides consisting of -D-glucan and no other type of homo- and hetero-polysaccharides (see Spanish patent application "PROCESS FOR MOLECULAR DETECTION OF LACTIC ACID BACTERIA PRODUCERS OF -GLUCANS" (2004)).

[0018] So, an object of the present invention consists of a DNA sequence, hereinafter GTF DNA sequence of the present invention, coming from GRAS lactic acid bacteria and encoding a protein with glycosyltransferase activity which permits the production of exopolysaccharides and which consists of one of the nucleotide sequences selected from between:

- a) the nucleotide sequence identified as SEQ ID NO9 or a fragment thereof; and
- b) a nucleotide sequence analogous to the sequence defined in a).

[0019] In the sense used in this description, the term "analogous" intends to include any DNA sequence that can be isolated or constructed on the basis of the sequence of nucleotides shown in SEQ. ID NO9, for example, by means of introducing conservative or non-conservative substitutions of nucleotides, including the insertion of one or more nucleotides, the addition or one or more nucleotides at either end of the molecule or the deletion of one of more nucleotides at either end or within the interior of the sequence.

[0020] In general, an analogous DNA sequence is substantially homologous with the sequence of nucleotides identified as SEQ ID NO9. In the sense used in this description, the expression "substantially homologous" signifies that the nucleotide sequences in question have a degree of identity at the nucleotide level of at least 60%, preferably at least 85%, and more preferably at least 95%.

[0021] The GTF DNA molecule of the invention comes from *P. damnosus* 2.6 and can be found in similar forms in other micro-organisms, among others, by way of illustration and without limiting the scope of the invention: *Lactobacillus* sp. and *Oenococcus oeni*, where they can be natural or they could otherwise be the result of a gene transformation process in which the transformed organism reproduces those DNA molecules. The DNA sequence of the invention can be isolated by means of conventional techniques starting from the DNA of any micro-organism containing it, by means of using probes or oligonucleotides, prepared thanks to the information of the nucleotide sequence of that DNA molecule, provided in this invention by an expert in the subject. Results obtained by the same research team indicate that the production of -glucan by the strains *Pediococcus damnosus* 2.6, *Lactobacillus* sp G77 and *Oenococcus oeni* 077 require the expression of *gtf* gene, whose allelic forms are virtually identical in all strains, though their genome location and the elements regulating their expression are different in each strain (see patent application "PROCESS FOR MOLECULAR DETECTION OF LACTIC ACID BACTERIA PRODUCERS OF -GLUCANS" (2004)).

[0022] Moreover, starting from the GTF DNA sequence of the present invention, an expert in the subject can obtain other deletions or protein fragments derived from this GTF protein or from its homologous forms which maintain its glycosyltransferase activity. Therefore, the DNA molecule of the invention includes fragments of it which present said glycosyltransferase activity.

[0023] In a particular embodiment, the GTF DNA sequence of the invention is a DNA molecule of *P. damnosus* 2.6 and in particular SEQ ID NO9.

[0024] The GTF DNA sequence of the invention can in general be used in the generation of an expression vector which permits expression of these proteins with glycosyltransferase activity in a broad range of host cells.

[0025] Therefore, the invention also refers to a GTF expression vector which comprises the GTF DNA sequence of the invention, hereinafter the GTF expression vector of the invention, and which permits the transformation of micro-organisms or cells and the subsequent obtaining of micro-organisms capable of expressing the GTF protein (SEQ ID NO6) and which permits the host to acquire the capacity, or improve the already existing capacity, to produce EPS.

[0026] In general, the expression vector of the present invention comprises at least one GTF DNA sequence of the invention and at least one promoter which directs the transcription of the gene of interest, to which it is operatively linked, and other sequences necessary or appropriate for the transcription of the gene of interest and its regulation in time and place, for example, starting and finishing signals, cutting sites, polyadenylation signal, replication origin, transcriptional activities, transcriptional repressors, insertion sequences, etc. Examples of suitable expression vectors can be selected according to the conditions and needs of each specific case from among plasmids and cosmids with a bacterial replication origin so that it can be amplified in multicopy starting from the extra chromosomal elements in bacteria or integrative

vectors so that it can be amplified in monocopy starting from the chromosome in bacteria, along with a marker that can be used for selecting the transformed cells different from the gene of interest. The choice of vector will depend on the host cell into which it is going to be introduced later on. By way of example, the vector where said DNA sequence is introduced can be a plasmid which, when introduced in a host cell, becomes integrated into the genome of said cell and is replicated along with the chromosome of the host cell.

[0027] The vector of the invention can be obtained by conventional methods known to technicians in the field (Sambrook et al. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press N.Y.). A particular object of the present invention consists of the plasmid pGTF which contains the GTF DNA sequence of the present invention (see Example 2).

[0028] Another object of the present invention is the use of the GTF DNA sequence of the GTF expression vector in a cell transformation process in order to obtain GTF cells.

[0029] A further object of the invention consists of a protein with glycosyltransferase activity which consists of one of the sequences of amino-acids selected from between:

- a) the sequence of amino-acids identified as SEQ ID NO6 or a fragment thereof; and
- b) a sequence of amino-acids analogous to the sequence defined in a).

[0030] Another object of the invention consists of a cell, hereinafter the GTF cell of the present invention, which comprises a sequence of GTF DNA or a pGTF expression vector of the invention, capable of expressing in recombinant form the GTF protein (SEQ ID NO6) and which therefore acquires the capacity, or improves the already existing capacity, to produce EPS.

[0031] The host cells that can be transformed with that expression vector can be both Gram-positive and Gram-negative bacterial cells, by way of illustration and without limiting the scope of the present invention, LABs belonging to the genera *Lactococcus*, *Lactobacillus* and *Streptococcus* such as *S. thermophilus* various subspecies of *L. lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which are currently used in the production of dairy products.

[0032] A particular embodiment of the present invention consists of the GTF cell *L. lactis* MG1363[pGTF] and the cell *S. pneumoniae* R61[pGTF].

[0033] The GTF cells of the invention can be used for the overproduction of EPSs for their subsequent use in the different possible applications. So, another object of the present invention is the use of the GTF cells for producing EPSs by means of a procedure comprising:

- a) growth of the GTF cell in a suitable medium and under conditions which permit expression of GTF expression vector or of GTF DNA sequence,
 - b) the production of EPSs by GTF cell of a), and
 - c) purification of the EPSs of b)
- Purification of the EPSs produced by GTF cells can be carried out by conventional techniques known to an expert in the subject.

[0034] As stated earlier, the EPSs thus obtained can be used as stabilising agents, thickeners, gelling agents and emulsifiers in many different processes of handling, processing and transformation of foods, such as for example as additives in the manufacture of numerous foods such as dairy derivatives (yoghurts, shakes and ice-creams), fruit juices and cereal-based preparations, as substitutes for cream and vinegar (Sutherland (1998) Trends Biotechnol. 16: 41-46), as stabilising agents for foods (Sutherland (1998) Trends Biotechnol. 16: 41-46), in cosmetics in rubber form as a thickener, stabiliser and suspension agent (Becker et al. (1998) Appl. Microbiol. Biotechnol. 50: 145-152) and also in the synthesis of matrices employed in chromatography techniques for coating metal surfaces or for stabilising syrups.

[0035] On the other hand, these results enable new possibilities to be opened up for transforming a GRAS bacterial system, in other words, micro-organisms among others, and by way of example without limiting the scope of the present invention, strains of LAB belonging to genera of the following group: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* - which can be used in the food industry in fermentation processes for foods, among others, and by way of example without limiting the scope of the present invention, those belonging to the following group: dairy products, alcoholic drinks and oats, which permit improvement of the texture of the fermented products. e.g., yoghurt and cheese (de Vos (1999) Cur. Opin. Biotechnol. 10: 483-484). or the improvement of their organoleptic qualities (Olof Mårtensson et al. (2002) Nutrition Research 22: 1461-1473).

[0036] Finally, a further object of the invention consists of the use of the GTF cells in fermentation process for foods, among others and by way of illustration without limiting the scope of this invention, those belonging to the following group: dairy products, alcoholic drinks and oats.

DESCRIPTION OF THE FIGURES

[0037]

Figure 1.- Physical map of pGTF plasmid. The genetic symbols used are: *gtf*, encodes the glycosyltransferase; *gfp* encodes the green fluorescent protein; *erm* encodes the protein responsible for erythromycin resistance; *malR* encodes the transcriptional repressor MalR; P_M, promoter of *gtf* and *gfp*, *copG* and *repB* encode the proteins implicated in plasmid replication.

Figure 2.- Prediction of the secondary structure of GTF protein from *P. damnosus* 2.6 using the SOSUI program. The colours of the amino-acids correspond to: black, hydrophobic; blue, with polar chain; blue and black, positively charged; red; negatively charged.

Figure 3.- Detection in *S. pneumoniae* and in *L. lactis* of *gtf* and *gfp* expression respectively by microscopic detection of agglutination and by fluorescence microscopy. Contains photographs of cell cultures of *S. pneumoniae* R61[pLS1RGFP] (A and B) and R61[pGTF] (C and D) and of *L. lactis*: MG1363[pLS1RGFP] and MG1363 [pGTF] detected by phase contrast microscopy (A, C, E and G) and by fluorescence microscopy (B, D, F and H).

EXAMPLES OF EMBODIMENT

Example 1: Cloning and characterisation of *gtf* gene from *P. damnosus* 2.6.

[0038] The cloning of the 5' region of the gene was carried out by PCR amplification using the total DNA of the plasmids carried by *P. damnosus* 2.6 and the degenerate oligonucleotides 5'-TAYGAYAAYACNCARGARGT-3' (Oligo I, SEQ ID NO1) and 5'-ACRAARTARTCARTTCRTG-3' (Oligo II, SEQ ID NO2) (Y = T or C; W; R = A or G; N = A, C, G or T). These oligonucleotides were designed on the basis of the amino-acid sequence conserved from the glycosyl transferase of *P. damnosus* IOEB8801 (Walling et al. (2001) Lait 81: 289-300) since the nucleotide sequence of the coding gene has not been published, nor deposited in the DNA sequence data banks. The amplified fragment of 1.8 kb was cloned in the vector pCR 2.1-TOPO (Invitrogen) and the recombinant plasmid obtained was established in *Escherichia coli* DH5. The determination of the nucleotide sequence of the DNA fragment revealed the existence of an open reading frame lacking its amino terminal region. The DNA sequence obtained was used for synthesising the oligonucleotides 5'-ACGCCCTGCGTGTTCATA-3' (SEQ ID NO3) and 5'-TGTGTAATGGCACTCACGAC-3' (SEQ ID NO4) and by means of reverse PCR reaction the 5' end of the *gtf* gene was amplified, which was cloned using the same vector, method and host bacterium as were used for cloning the 3' end of the gene. The sequence of 3352 nucleotides of the cloned regions is shown in SEQ ID NO5 (nucleotides 1-621 of the 3' region of the *mobA* gene, nucleotides 819-1085 of the hypothetical ORF1 gene and nucleotides 1282-2982 of the *gtf* gene -CDS region). The *gtf* gene (SEQ ID NO 9) encodes the GTF protein of 567 amino acids detailed in SEQ ID NO6.

[0039] The comparison of the DNA sequence of *gtf* gene from *P. damnosus* with those deposited in databases shows the absence of significant homologies. Nevertheless, when comparing the protein sequence (with the Swissprot database) encoded by the *gtf* gene (GTF) it was observed that it is homologous to the glycosyltransferases of the COG1215 family. Also, the GTF protein presents a relatively high (34%) homology with the Tts protein of *Streptococcus pneumoniae*, a glycosyltransferase which is the only enzyme required for the biosynthesis and secretion of -D-glucan similar to that of *P. damnosus* (Llul et al. (1999) J. Biol. Chem. 24: 21053-21061). This feature contrasts with the operon complexes (consisting of ten genes) which encode proteins required for the synthesis and secretion of bacterial heteropolysaccharides and homopolysaccharides currently used at industrial level (Jolly and Stingle (2001) Int. Dairy J. 11: 733-745). As a consequence, a protein with features similar to Tts, but coming from a non-pathogenic micro-organism having food quality would have potential for the production of -D-glucans by genetic manipulation and would be of industrial interest.

[0040] Results obtained by the same research group indicate that production of -glucan by *Pediococcus damnosus* 2.6, *Lactobacillus* sp G77 and *Oenococcus oeni* 077 strains, require the expression of the *gtf* gene, whose allelic forms are virtually identical in all strains, though their genomic location and elements regulating their expression are different in each strain (see patent application "PROCESS FOR MOLECULAR DETECTION OF LACTIC ACID BACTERIA PRODUCERS OF -GLUCANS" (2004)).

Example 2: Construction of the pGTF vector which permits overexpression of GTF protein of *Pediococcus damnosus* 2.6.

[0041] As already mentioned earlier, the homology of GTF with Tts glycosyltransferase of *S. pneumoniae* (Llul et al. (1999) J. Biol. Chem. 24: 21053-21061) and which constitutes the capsule of the serotype 37 (Llul et al. (1999) J. Biol. Chem. 24: 21053-21061) indicated that GTF is possibly the only protein responsible for biosynthesis of the exopolysaccharide of *P. damnosus*. For this reason, as part of this invention, the recombinant plasmid pGTF overproducer of GTF

and which contains the DNA sequence of the gene *gtf* of the invention (SEQ ID NO9) was constructed (Figure 1). In the expression vector pLS1RGFP (Nieto et al. (2000) Plasmid 43: 205-213) between the inducible promoter P_M and the *gfp* reporter gene a fragment of DNA was cloned which includes the coding region of the *gtf* gene. To do this, the region which includes the *gtf* gene was amplified using Pfu DNA polymerase (Stratagene) and using as substrate the total DNA of the plasmids carried by *P. damnosus* 2.6 and the oligonucleotides 5'-TCTAGAAATTAAAGGAATGTGTAA-3' (SEQ ID NO7) and 5'-TCTAGATTAATCATTCCAATCAACTG-3' (SEQ ID NO8), which include the recognition sequence for restriction enzyme XbaI. The amplified fragment of 1.734 kb was cloned in the correct orientation with respect to P_M promoter in the XbaI unique site of pLS1RGFP and, subsequently, the recombinant plasmid was established in the non-capsulated strain R61 of *S. pneumoniae* (strain R6 whose genome has been sequenced by Hoskins et al. (2001) J. Bacteriol. 183: 5709-5717 and which was named R61 by Dr Sanford Lacks in: Lacks (1968) Genetics 60: 685-706) by transformation and selection of transformants due to resistance to 5 g ml⁻¹ of erythromycin (resistance marker of the plasmid vector) as described in Lacks (1968) Genetics 60: 685-706), obtaining the strain *S. pneumoniae* R61[pGTF]. The pGTF plasmid, which is capable of replicating in Gram-positive and Gram-negative bacteria, contains a transcriptional fusion P_M -*gtf*-*gfp*, which permits an overexpression by growth of the carrier cells in medium containing maltose and a detection of the expression from the promoter P_M by measurement of GFP protein fluorescence (Nieto et al. (2000) Plasmid 43: 205-213).

Example 3.- *In vitro* detection of GTF function integrated in *S. pneumoniae* membranes

[0042] Glycosyltransferase Tts of *S. pneumoniae* is an integral membrane protein (Llul et al. (1999) J. Biol. Chem. 24: 21053-21061). The analysis of the prediction of topological location of GTF from *P. damnosus* 2.6 using the SOSUI program (<http://sosui.proteonem.bio.tuat.ac.jp/sosui/frame0.html>) revealed that the catalytic domain of the protein (amino-acids 83 to 377) is located in the bacterial cytoplasm and that there exist six transmembrane regions: two located at the amino terminal end of the protein preceding the catalytic domain of it and four located in its carboxylic region (Figure 2). For the reasons mentioned earlier, membranes of *S. pneumoniae* R61 strains carriers of the pGTF plasmid or of the pLS1RGFP plasmid vector were purified and the glycosyltransferase activity was determined using UDP-glucose as substrate. To do this, cells of the strains of *S. pneumoniae* R61 [pLS1RGFP] (Nieto et al. (2000) Plasmid 43: 205-213) and R61[pGTF] were grown in 1 l of Tood-Hewitt medium (Difco) supplemented with 0.5% yeast extract, 0.1% saccharose and 0.8% maltose to an A_{650} of 0.4. The cultures of both strains showed a similar duplication time (Table 1), indicating that expression of the P_M -*gtf*-*gfp* fusion did not have any deleterious effect on *S. pneumoniae*. The assessment of the expression from P_M promoter was carried out by means of fluorescence determination due to the GFP protein of 0.1 ml of the cultures by means of fluorescence spectroscopy (Nieto et al. (2000) Plasmid 43: 205-213). The results obtained (Table 1) showed that in both strains analysed there existed GFP protein expression that was slightly less in R61[pGTF] than in the strain carrying the vector, an effect that was observed in constructions prior to inserting a gene between the P_M promoter and the *gfp* gene (results not shown).

[0043] In order to obtain the membranes required for determining glycosyltransferase activity, the cells present in 1 litre of medium were sedimented by centrifugation at 12000 x g for 20 minutes (min) at 4°C and resuspended in buffer A (70 mM Tri.HCl pH 7.0, 9 mM MgCl₂, 1 mM CaCl₂) with 0.2 M phenylmethylsulphonyl fluoride (PMSF) and sedimented by centrifugation at 10000 x g for 10 min at 4°C. The bacteria resuspended in buffer A were subjected to mechanical rupture by passing them twice through a French press (Aminco). The homogenised product was centrifuged at 12000 x g for 15 min at 4°C and the supernatant was again centrifuged at 12000 x g for 30 min at 4°C. Sedimented membranes were once more resuspended in 2 ml of buffer A containing 0.2 mM of PMSF and stored at -80°C. Protein concentration of the membrane preparations was determined using the BCA Protein Assay system (Pierce). Determination of the glycosyltransferase activity was done using 30 M (0.1 Ci) UDP-[¹⁴C]glucose (specific activity of 333 mCi/mmol) in a buffer A supplemented with 50 mM NaCl in a total volume of 100 l and membranes in a range of protein concentrations between 0.05 and 0.5 mg ml⁻¹. The reactions were conducted by incubation at 30°C for 15 min. The reaction was terminated by addition of SDS to a final concentration of 0.5% and incubation at 37°C for 15 min. Bovine seroalbumin was then added to a final concentration of 0.4% along with 1 ml of 10% trichloroacetic acid (TCA). Following incubation for 30 min at 0°C, the mixtures were filtered through Wathman GF/A filters and were washed with 15 ml of 10% TCA. The filters were dried at 65°C for 20 min and the radioactivity present in them was measured in a scintillation counter (LKB Wallack). The results obtained (Table 1) revealed the existence of glycosyltransferase activity in the membranes of the two strains analysed. The levels were approximately 2.5 times higher in strain R61[pGTF] in comparison with the control strain. This increase was presumably due to the glycosyltransferase activity of the GTF from *P. damnosus*. In the case of control strain R61 [pLS1RGFP] the levels of activity detected could be due to the products of chromosomal genes *cpoA* and *epsG* of *S. pneumonias*, whose function is unknown, but by homology they are proposed as glycosyltransferases in the KEGG data bank (<http://www.genome.jp/kegg/>).

Table 1. Effect: of the induction of the fusion P_M -*gtf-gfp* on the growth, GFP levels and glycosyltransferase activity in *S. pneumoniae*

Strain	¹ Duplication time (min)	² GFP fluorescence (arbitrary units)	³ glycosyl-transferase activity (units)
R61 [pLS1RGFP]	30	152.85	243.71 ± 42
R61 [pGTF]	35	98.7	615.46 ± 193

¹ The duplication time of the bacteria was calculated using the measurement of A_{650} during growth.
² The fluorescence of 0.1 ml of the cultures previously sedimented and resuspended in 0.1 ml of PBS buffer pH 7.2 was measured on micro-titration plates in an LS_50B spectrophotometer (Perkin Elmer) by means of excitation at a wavelength of 488 nm with an aperture of 5 and the detection of fluorescence was done at a wavelength of 511 nm with an aperture of 5.
³ Values represent the mean and standard deviation of 10 determinations using different membrane concentrations in the range 0.05 to 0.5 mg ml⁻¹ of protein. One unit of glycosyltransferase activity corresponds to the quantity of enzyme which catalyses the incorporation of a macromolar product of 1 pmol of glucose per mg of protein and per minute.

Example 4.- Detection of GTF function *in vivo* in *S. pneumoniae* and *L. lactis*

[0044] The results of detection of glycosyltransferase activity indicated that pGTF plasmid encodes the active GTF protein. Since antibodies developed against the capsule of serotype 37 of *S. pneumoniae* are very specific and do not react with non-capsulated strains or with other serotypes of *S. pneumoniae* or with other bacteria, these antibodies were used in order to make an *in vivo* assessment of the functional expression of GTF from *Pediococcus* both in *S. pneumoniae* R61[pGTF] and in the strain MG1363 of *Lactococcus lactis* (to which the plasmid pGTF had been transferred by transformation). Functional expression of GTF was determined by means of the agglutination technique using antibodies developed against the capsule of the serotype 37 of *S. pneumoniae* and by microscopy (Llul et al. (1999) J. Biol. Chem. 24: 21053-21061). Cells from *S. pneumoniae* strains R61[pLS1RGFP] and R61[pGTF] were grown in Tood-Hewitt medium (Difco) supplemented with 0.5% yeast extract, 0.1% saccharose and 0.8% maltose to an A_{650} of 0.4. Cells from the *L. lactis* strains MG1363[pLS1RGFP] and MG1363[pGTF] were grown in Tood-Hewitt medium (Difco) supplemented with 0.5% yeast extract, and 5% maltose to an A_{660} of 0.5. One ml of each of the cultures was sedimented by centrifugation and cells were resuspended in 100 μ l of PBS buffer pH 8.0 (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 140 mM NaCl, 3 mM KCl). Ten μ l of each of the cultures were mixed with 10 μ l of antibody developed against pneumococcus serotype 37 (Statens Seruminstitut, Denmark) and kept at 4°C for 2 hours. The preparations were analysed by phase contrast and fluorescence microscopy using a Zeiss Axioplan microscope (Universal microscope) with excitation fluorescence filters Standard FITC set D480/30 and emission TBP 460/530/610. The results obtained appear in Figure 3. The fluorescence of GFP protein permitted the detection by fluorescence microscopy of the existence of gene expression starting from the P_M promoter of pLS1RGFP and of pGTF in the cells of *S. pneumoniae* (Figures 3B and 3D) and *L. lactis* (Figures 3F and 3H) induced with maltose. The images obtained by fluorescence and phase contrast microscopy revealed that only the cells of *S. pneumoniae* (Figures 3C and 3D) and of *L. lactis* (Figures 3G and 3H) that were carriers of the pGTF plasmid and induced with maltose were capable of agglutination. These results show that pGTF confers *S. pneumoniae* and *L. lactis* with the capacity to synthesise the exopolysaccharide.

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	attccgggtt gttttgggtt ttgtttcgaa atgcaagcta atcatcggaac atcagaagaa	1620
	caaataaaag cggataaatt acagagttac aacataaagg cattacaatc tggcaacacg	1680
55	gaaacagttg attggaatga t	1701

Claims

1. GTF DNA sequence **characterised in that** it comes from GRAS lactic acid bacteria, it encodes a protein with glycosyltransferase activity which permits the production of exopolysaccharides (EPSs) and **in that** it consists of one of the nucleotide sequences selected from between:
 - a) the sequence of nucleotides identified as SEQ ID NO9 or a fragment thereof; and
 - b) a sequence of nucleotides analogous to the sequence defined in a).
2. GTF DNA sequence according to claim 1 **characterised in that** it is the SEQ ID NO9.
3. GTF expression vector **characterised in that** it comprises the GTF DNA sequence according to claims 1 and 2.
4. GTF expression vector according to claim 3 **characterised in that** it is the pGTF plasmid.
5. Use of the GTF DNA sequence according to claims 1 and 2 or of the GTF expression vector according to claims 3 and 4 in a process of cell transformation.
6. Protein with glycosyltransferase activity **characterised in that** it consists of one of the sequences of amino-acids selected from between:
 - a) the sequence of amino acids identified as SEQ ID NO6 or a fragment thereof; and
 - b) a sequence of amino acids analogous to the sequence defined in a).
7. GTF cell **characterised in that** it comprises a GTF DNA sequence according to claims 1 and 2 or a pGTF expression vector according to claims 3 and 4, capable of expressing in recombinant form the GTF protein according to claim 6 and therefore acquires the capacity, or improves the already existing capacity, to produce EPSs.
8. Use of GTF cells according to claim 7 in a procedure for producing EPSs.
9. Use of GTF cells according to claim 8 **characterised in that** the procedure comprises:
 - a) the growth of the GTF cell in a suitable medium and under conditions which permit expression of the GTF expression vector or of the GTF DNA sequence,
 - b) the production of EPSs by the GTF cell, and
 - c) purification of the EPSs of b).
10. Use of the GTF cells according to claim 7 in food fermentation processes.
11. Use of the GTF cells according to claim 10 **characterised in that** the fermentation processes for foods belong to the following group: dairy products, alcoholic drinks and oats.

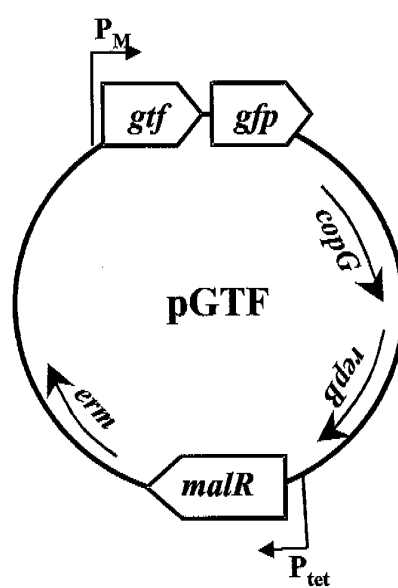


Figure 1

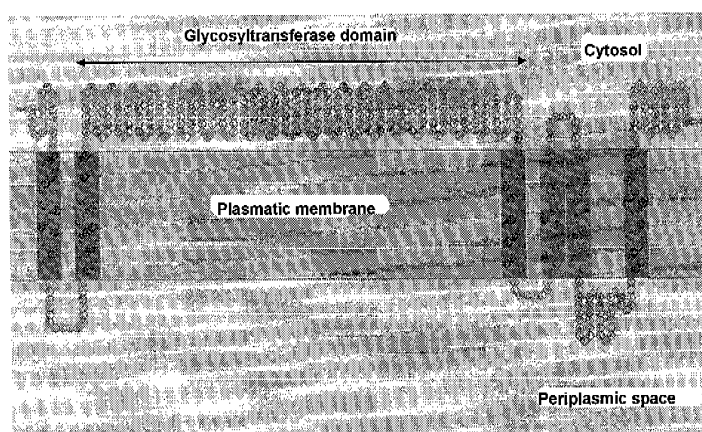


Figure 2

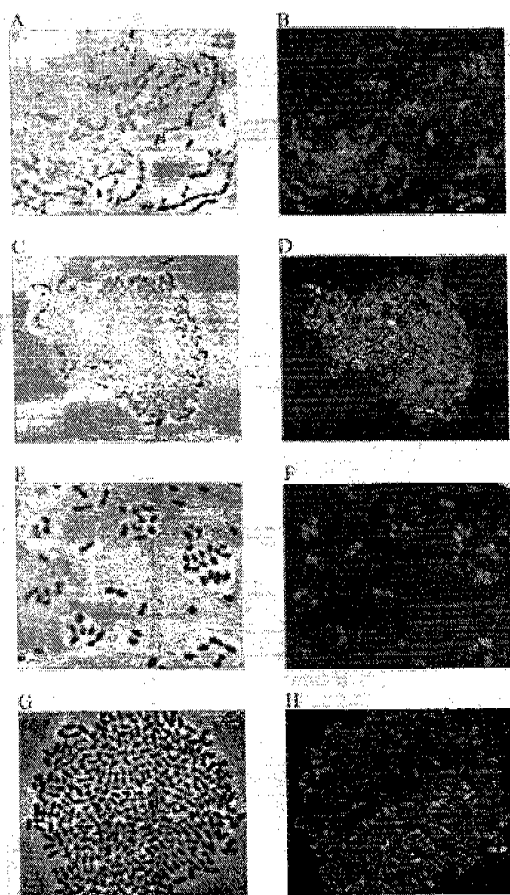


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/ ES 2005/070127

A. CLASSIFICATION OF SUBJECT MATTER		
C12N 9/10 (2006.01)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
CIBEPAT, EPODOC, WPI, MEDLINE, BIOSIS, NPL, XPESP, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALLING, E., GINDREAU, E., LONVAUD-FUNEL, A. La biosynthèse d'exopolysaccharide par des souches de <i>Pediococcus damnosus</i> isolées du vin: mise au point d'outils moléculaires de détection. Lait. Enero-abril 2001, Vol. 81, N° 1-2, pages 289-300. ISSN 0023-7302.	1-9
A		10, 11
A	MARTENSSON, O., STAAF, M., DUEÑAS-CHASCO, M. et al. A fermented, ropy, non-dairy oat product based on the exopolysaccharide-producing strain <i>Pediococcus damnosus</i> . Advances in Food Sciences. 2002, Vol. 24, N°1, pages 4-11. ISSN 1431-7737.	8-11
A	MARTENSSON, O., HASKA, L., DUEÑAS-CHASCO, M., et al. Development of an oat-based sour milk-like product. Advances in Food Sciences. 2003, Vol. 25, N°3, pages 100-106. ISSN 1431-7737.	8-11
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 16 December 2005 (16/12/2005)		Date of mailing of the international search report 22 December 2005 (22/12/2005)
Name and mailing address of the ISA/ S.P.T.O. C/Panamá 1, 28071 Madrid, España. Facsimile No. N° de fax 34 91 3495304		Authorized officer E. Relaño Reyes Telephone No. 34 91 3493047

Form PCT/ISA/210 (second sheet) (July 1992)

REFERENCES CITED IN THE DESCRIPTION

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