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(54) **PRODUCTION D'EXOPOLYSACCHARIDE DE BACTERIES
NON NATIVES DANS UN HOTE BACTERIEN RECOMBINE**
(54) **PRODUCTION OF NON-NATIVE BACTERIAL
EXOPOLYSACCHARIDE IN A RECOMBINANT BACTERIAL
HOST**

(57) L'invention concerne une bactérie recombinée utile pour la production d'exopolysaccharides, ainsi qu'un procédé permettant de produire la bactérie recombinée et un exopolysaccharide provenant de la bactérie par fermentation aérobique immergée de la bactérie au moyen d'un substrat de sucre. De plus, la présente invention concerne un procédé permettant de produire des exopolysaccharides de bactéries, par fermentation à partir de substrats de sucre que la bactérie de type sauvage servant à produire l'exopolysaccharide ne peut pas utiliser.

(57) A recombinant bacteria for the production of exopolysaccharides is disclosed as well as a method for making the recombinant bacteria and making an exopolysaccharide from the bacteria by submerged aerobic fermentation of the bacteria utilizing a sugar substrate. In addition, the present invention provides a method of producing bacterial exopolysaccharides by fermentation from sugar substrates that the wild-type bacteria for producing the exopolysaccharide cannot utilize.

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(54) Title: PRODUCTION OF NON-NATIVE BACTERIAL EXOPOLYSACCHARIDE IN A RECOMBINANT BACTERIAL HOST (57) Abstract A recombinant bacteria for the production of exopolysaccharides is disclosed as well as a method for making the recombinant bacteria and making an exopolysaccharide from the bacteria by submerged aerobic fermentation of the bacteria utilizing a sugar substrate. In addition, the present invention provides a method of producing bacterial exopolysaccharides by fermentation from sugar substrates that the wild-type bacteria for producing the exopolysaccharide cannot utilize.		

PRODUCTION OF NON-NATIVE BACTERIAL EXOPOLYSACCHARIDE IN A RECOMBINANT BACTERIAL HOST

BACKGROUND OF THE INVENTION

Xanthan gum is an acidic exopolysaccharide (EPS) normally secreted by *X. campestris* (Jeanes, A, et al., 1961, J Appl Polymer Sci 5: 519-526), and is useful as an aqueous rheological control agent because it exhibits high viscosity at low concentration, pseudoplasticity, and insensitivity to a wide range of temperature, pH, and electrolyte conditions (see U.S. Patents 5,194, 386, 5,472,870, 5,279,961, 5,338,841, and 5,340,743, the contents of each of which are incorporated herein by reference). The genes that code for its synthesis are *gumB* through *M* (Capage, MA, et al., 1987, WO87/05938; Vanderslice, RW, et al., 1989, the contents of which are incorporated by reference; Genetic engineering of polysaccharide structure in *Xanthomonas campestris*. In: Biomedical and biotechnological advances in industrial polysaccharides, V Crescenzi, ICM Dea, S Paoletti, SS Stivala, and IW Sutherland, eds, pp 145-156, Gordon and Breach Science Publishers, New York).

A different source of commercially significant and functionally diverse biopolymers is the genus *Sphingomonas* (Pollock, TJ, 1993, J Gen Microbiol 139: 1939-1945). Different organisms of this genus secrete a variety of different strain-specific exopolysaccharides. For example, one species secretes Gellan®, while others secrete welan, rhamsan, S-88 or other polysaccharides (Moorhouse, R, 1987, Structure/property relationships of a family of microbial polysaccharides. In: Industrial polysaccharides: genetic engineering, structure/property relations and applications. M Yalpani, ed, pp 187-206, Elsevier Science Publishers B.V. Amsterdam).

We refer to this group of polymers as "sphingans," after the common genus, because they also have common carbohydrate backbone structures (-x-glucose-glucuronic acid-glucose-; where x is either L-rhamnose or L-mannose) with distinct side chains. (See U.S. Patent Applications serial numbers 08/592,874, filed January 24, 1996, and

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08/377,440, filed January 24, 1995, the contents of each of which are hereby incorporated by reference). The structure for sphingan S-88 is shown in Figure 1. The organization and DNA sequence of 23 genes (Figure 2) that direct the synthesis of sphingan S-88 have been described (Yamazaki, M, et al., 1996, J Bacteriol 178: 2676-2687).

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The commercial production of highly viscous xanthan gum and other bacterial polysaccharides is a complex biosynthetic and process-engineering problem (Kennedy, JF et al., 1984, Prog Industrial Microbiol 19: 319-371). The sugar substrate is important primarily because the sugar affects productivity, but the cost of the sugar can also be significant. Currently, xanthan gum is produced by supplying *X. campestris* with corn syrup, sucrose or starch. Yet, three to four typical cheese factories can provide enough low-value lactose-containing waste whey to satisfy all of the existing worldwide demand for xanthan production.

A recombinant strain that can stably convert lactose into xanthan gum in amounts equal to the conversion of glucose is disclosed in U.S. Patents 5,434,078, and 5,279,961, the contents of each of which are incorporated herein by reference.

It is desired to improve the methods of the production of xanthan gum to achieve more cost-effectiveness, convenience, more desired product qualities and greater production efficiency.

A problem encountered with xanthan gum produced by conventional methods, is that it is contaminated with a cellulase which can be very disadvantageous in commercial applications where xanthan is mixed with or contacts cellulosic polymers. The result is deterioration of the cellulosic polymers.

Methods are known for the treatment of xanthan gum which has been separated from fermentation broths to remove the cellulase contaminant. However, these treatments require processing of the xanthan gum and add to the expense and overall complexity of the process.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the repeating subunit structures of xanthan gum and sphingan S-88.

5 Figure 2 is a schematic map of the *X. campestris* gum and *Sphingomonas* S88 *sps* genes.

SUMMARY OF THE INVENTION

We have discovered new recombinant bacteria for the production of exopolysaccharides. In addition, we have discovered a method for making the recombinant
10 bacteria and making an exopolysaccharide from the bacteria by submerged aerobic fermentation of the bacteria utilizing a sugar substrate. The recombinant bacteria of the present invention are able to produce exopolysaccharides and utilize sugar substrates which are utilizable by the bacteria from which the recombinant bacteria were derived. In addition, the exopolysaccharide obtained from the inventive bacteria exhibit improved,
15 more desirable or different properties from the exopolysaccharide produced by the non-recombinant bacteria from which the recombinant bacteria was derived.

In addition, we have discovered a novel xanthan gum product which as obtained in the broth exhibits reduced cellulase contamination. It is an object of the invention to provide a method of producing bacterial exopolysaccharides by fermentation
20 from sugar substrates that the bacteria which the exopolysaccharides are native to cannot utilize.

It is a further object of the invention to provide a method of increasing the yield of a non-native bacterial exopolysaccharide produced in a recombinant bacterial host.

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It is another object of the invention to produce xanthan gum by fermentation from whey waste, a byproduct of cheese production.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may
5 be learned from practice of the invention. The objects and advantages may be realized and attained by means of the methods particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, methods for the production of non-native bacterial exopolysaccharides in recombinant bacterial hosts are set forth.

10 Specifically, we have discovered that the yield of a non-native bacterial exopolysaccharide produced in a recombinant bacterial host can be increased by inactivating the native polysaccharide production in the bacterial host.

DEFINITIONS

As used herein:

15 "Non-recombinant bacterial host strain" means a bacterial strain which does not contain foreign genetic material.

"Recombinant bacterial host strain" means the non-recombinant bacterial host strain into which foreign genetic material has been introduced and retained. This strain is sometimes referred to herein as the "recipient" or "recipient strain."

20 "Foreign genetic material" means segments of the genome of a strain of bacteria which are different from those in the recombinant bacterial host strain into which the segment(s) is or is to be introduced.

"Glycosyl transferase" means any one of a group of related enzymes which either catalyze the attachment of a sugar-phosphate molecule to the isoprenyl phosphate carrier
25 involved in exopolysaccharide biosynthesis or the attachment of a sugar to sugars previously attached to the isoprenyl phosphate carrier.

An "sps gene" means one of several genes which are present in the genomes of species of *Sphingomonas* bacteria or can be isolated from the *Sphingomonas* bacteria and

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which are involved in the biosynthesis of a sphingan exopolysaccharide because they code for enzymes that catalyze chemical reactions in the biosynthetic pathway or because they code for proteins or DNA control sites that modulate the amount of sphingan exopolysaccharide present in the bacterial growth medium.

5 "Non-native exopolysaccharide" means a bacterial exopolysaccharide which is not produced and excreted by a non-recombinant bacterial host strain, but is produced and excreted by a recombinant bacterial host strain obtained from the non-recombinant bacterial host strain.;

"Native bacterial producer" means a non-recombinant bacterial strain which produces the
10 desired bacterial exopolysaccharide.

A "gum gene" means one of several genes which are present in the genomes of species of *Xanthomonas* bacteria or can be isolated from the *Xanthomonas* bacterial and which are involved in the biosynthesis of a xanthan exopolysaccharide because they code for enzymes that catalyze chemical reactions in the biosynthetic pathway or
15 because they code for proteins or DNA control sites that modulate the amount of xanthan exopolysaccharide present in the bacterial growth medium.

Examples of two exopolysaccharides , xanthan gum and sphingan S-88 (Jansson, P-E, et al., 1975, Carbohydr Res 45: 275-282, Jansson, P-E, et al., 1986, Carbohydr Res 156: 165-172), are shown by their repeating sugar subunit structures in
20 Figure 1. The arrows point toward the reducing end of each repeat. For xanthan gum the IP carrier is attached at the reducing end through a phosphodiester linkage to the glucose residue which is lacking the side chain (Ielpi, L, et al., 1993, J Bacteriol 175: 2490-2500). Abbreviations: Glc, glucose; Man, mannose; GlcA, glucuronic acid; Rha, rhamnose; Ac, acetyl ester; and Pyr, acetal-linked pyruvic acid. The position and linkage of the Ac in S-
25 88 is unknown.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method of producing a bacterial exopolysaccharide from a sugar source which the native bacterial producer of that

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exopolysaccharide cannot utilize by transforming a bacterial host strain which can utilize the sugar source but does not produce the exopolysaccharide with genes from the native bacterial producer which are necessary for establishing the production of the exopolysaccharide in the bacterial host strain. The bacterial exopolysaccharide can then be produced by fermenting the recombinant bacterial host with the sugar source. For example, the genes *gumBCDEFGHIJKL* and *M* from *X. campestris* strain B1459 were transformed into a specifically mutated *Sphingomonas* recipient, fermentation of which in the presence of inexpensive waste whey lactose obtained large amounts of secreted xanthan gum which was comparable to that produced by *X. campestris* strain B1459. For production of xanthan gum lacking acetyl side groups, the *gumF* and *gumG* genes of *Xanthomonas* can be omitted from the foreign genetic material obtained from *Xanthomonas*. Similarly, for production of xanthan gum lacking pyruvyl side groups, the *gumL* gene can be omitted.

In the case where the "recombinant bacterial host strain" already expresses a gene function that is necessary for production of the "non-native exopolysaccharide", then that function need not be included in the "foreign genetic material". For example, when foreign genetic material is introduced into *Sphingomonas* bacteria and the initial step in assembly of the repeat subunit structure of the non-native polysaccharide on the isoprenoid lipid carrier is the transfer of glucose-P to the carrier, then any of several gene functions that carry out the same specific enzymatic reaction can be substituted. For example, in place of the *gumD* gene of *Xanthomonas* species one could substitute the *spsB* gene from *Sphingomonas* species or the *pssA* gene from *Rhizobium* species, or analogous genes from other bacteria.

The present invention further provides a method for increasing the yield of a non-native exopolysaccharide produced in a recombinant bacterial host strain by inactivating the exopolysaccharide production native to the bacterial host strain. Preferably, the inactivation is achieved by deletions or mutations in the genome of the bacterial host strain. Most preferably, the deletion or mutation inactivates the activity of a glycosyl transferase.

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Most preferably, the deletion or mutation inactivates the activity of a glycosyl transferase, a polysaccharide polymerase, a secretory apparatus, or enzymes required for the synthesis of essential nucleotide-sugar precursors. For example, in *Sphingomonas*, inactivation of one or more of the essential substrate-specific glycosyl transferase enzymes, such as, *spsB*, Q, K or L, which are required for synthesis of the subunit sugar repeat structure which is attached to the carrier C55-isoprenyl phosphate eliminates synthesis of the polysaccharide produced by *Sphingomonas*. Likewise, mutations in a polysaccharide polymerase or in a secretion apparatus also eliminate exopolysaccharide production by a native polysaccharide producer. Production of the native polysaccharide can also be eliminated by mutations which inactivate enzymes that are essential for the synthesis of precursor nucleotide-sugars, such as the four enzymes required to synthesize the precursor dTDT-L-rhamnose:RhsA, RhsB, RhsC, or RhsD. The mutations or deletions which eliminate these enzyme or protein activities can either directly inactivate these enzymes by altering the structures and activities of the enzymes or indirectly inactivate the enzymes by blocking or modulating the expression of the genes that code for these enzymes or proteins.

The present invention also provides a method for minimizing contaminating cellulase activity in xanthan gum produced by fermentation by fermenting a recombinant *Sphingomonas* species strain transformed with genes from the genome of *X. campestris*, which are necessary for establishing the production of xanthan gum in the *Sphingomonas* species.

All of the embodiments use the following bacterial strains, recombinant DNA procedures, and growth media:

Strain X59 (ATCC 55298 - see U.S. Patent 5,194,386, col. 8-9) is a spontaneous rifampicin-resistant mutant derived from wild type *X. campestris* B1459 (Thorne, L, et al., 1987, J Bacteriol 169: 3593-3600). Cloned DNA segments and site-specific chromosomal deletions are diagramed in Figure 2. Plasmid XCc8 is a member of a cosmid library (Thorne, L, et al., 1987, J Bacteriol 169: 3593-3600) and was obtained by

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inserting a segment of about 24 kilobase pairs (kbp) of chromosomal DNA from strain X59 into the mobilizable broad-host-range plasmid vector pRK311 (Ditta, G, et al., 1985, Plasmid 13: 149-153). The DNA segment carried by plasmid XC600(*gumB-M*) was derived from plasmid XCc8 by standard methods of DNA isolation, digestion with
5 restriction endonucleases, and ligation (Maniatis, T, et al., 1982, Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor). The insert in plasmid XC600 spans the NdeI-SalI segment of XCc8 corresponds to nucleotide 919-15400 of the *gum* sequence in GenBank accession number U22511, shown in Sec. ID No.1 hereof. (GenBank, National Center for Biotechnology Information, National Library of
10 Medicine, Bethesda, MD 20894, www.ncbi.nlm.gov/web/genbank). Plasmid XC1483 includes nucleotide 3460 (BamHI) through 7959 (BamHI) (Pollock, TJ, et al., 1994, J Bacteriol 176: 6229-6237). Plasmids S88c1, and S88c3 are members of a cosmid library (Yamazaki, M, et al., 1996, J Bacteriol 178: 2676-2687) that were derived from a partial digestion with SalI enzyme of S88 chromosomal DNA, followed by ligation to the vector
15 pRK311. The nucleotides deleted in strain S88ΔTn358 include 4485 (BamHI) through 24646 (EcoRI) in the S88 sequence (GenBank accession U51197 Sec. ID No. 2 hereof). Strain S88m265 is defective in *spsB* (Yamazaki, M, et al., 1996, J Bacteriol 178: 2676-2687).

DNA transformation into *E. coli*, tri-parental conjugal mating of broad-host-
20 range plasmids into *Sphingomonas* or *X. campestris*, and selection methods were described (Yamazaki, M, et al., 1996, J Bacteriol 178: 2676-2687).

Luria-Bertani and M9 salts are standard media (Pollock, TJ, et al., 1994, J Bacteriol 176: 6229-6237). M9+YE is M9 salts supplemented with 0.05 % w/v yeast extract. YM medium contains 3 g Bacto yeast extract, 3 g malt extract, 5 g Bacto peptone,
25 and 10 g D-glucose per liter of deionized water. 1/4 YM-G medium is YM diluted with 3 volumes of water and with no added glucose. The amounts of antibiotics used were as follows: rifampicin, 50 mg L⁻¹; streptomycin, 50 mg L⁻¹; kanamycin, 50 mg L⁻¹; and tetracycline, 6-15 mg L⁻¹ (Sigma Chemical Co.). Low viscosity carboxymethylcellulose (1% w/v final, Sigma Chemical Co.) was mixed with TSA blood agar base (Difco), and

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then cultures were spotted onto the surface of the medium and grown for 4-7 days at 30°C. Zones of hydrolysis of carboxymethylcellulose were observed by gently flooding the plate with 0.1% (w/v) Congo red dye for 30 min followed by destaining with 1M NaCl for 30 min. The diameters of the zones were then measured to compare the relative cellulase
5 activity of the various cultures.

The following chemical and physical analyses of exopolysaccharides were used in the examples:

Extracellular xanthan gum or sphingan S-88 was precipitated from liquid culture medium with 2-3 volumes of isopropyl alcohol, and then dried at 80°C before
10 weighing. Hydrolysis mixtures contained 0.5-5 mg of polysaccharide and 130-260 µl 2M (v/v) trifluoroacetic acid in high performance liquid chromatography (HPLC) water, and were incubated for 16 h at 95°C. The samples were dried under vacuum, resuspended in 100 µl HPLC water, dried again, and finally resuspended in 100 µl of HPLC water. Samples and sugar standards were separated on a CarboPac PA-1 anion-exchange column
15 and the sugar compositions were quantitated with a Dionex DX500 HPLC system as described previously (Clarke, AJ, et al., 1991, Anal Biochem 199: 68-74). Assays for acetyl (Hestrin, S, 1949, J Biol Chem 180: 249-261) and pyruvyl (Duckworth, M, et al., 1970, Chem Ind 23: 747-748) groups have been described.

Samples of polysaccharides (10 mg, powdered) were dissolved in 2 ml
20 deionized water at 80°C for 60 min in glass test tubes with an equal weight of locust bean gum and bromphenol blue dye (100 µg ml⁻¹). After cooling for 2 h the tubes were rotated to horizontal for photography. Failure to gel resulted in horizontal movement of the mixed slurry.

A sample of commercial xanthan gum (Keltrol, Kelco Company) was used
25 for physical and chemical comparisons.

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For xanthan-guar mixtures each polysaccharide was dissolved in 100mM KCl at 0.1% w/v, and solution viscosities were measured at 20-25 °C at several rpm with a Brookfield LVTDV-II viscometer and spindle 18.

DEPOSITS

5 The following deposits have been made in connection with the present invention:

1. *Sphingomonas paucimobilis* (ATCC 29837) containing plasmid XCc8
ATCC Designation No. 98479;
2. *Sphingomonas* strain S88ΔTn358 containing plasmid XCc8
10 ATCC Designation 98480.

The above deposits were made with the American Type Culture Collection located at 10801 University Blvd., Manassas, VA 20110-2209, pursuant to the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. All restrictions on the availability of the materials deposited will be
15 irrevocably removed upon the issuance of a patent thereon.

All other microorganisms and/or DNA segments, plasmids, and the like referred to herein are publicly available from the American Type culture collection in Manassas, Virginia.

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Example 1Inter-generic expression of genes coding for polysaccharide biosynthesis

Referring to Figure 2, maps of *X. campestris* gum and *Sphingomonas* S88 *sps* genes, boundaries of specific segments cloned in plasmids, and deletions in bacterial
5 chromosomes are shown. The arrows above the genes indicate the direction of transcription. The horizontal lines indicate the extent of the cloned DNA included in each plasmid with the included genes within parentheses. The dashed lines indicate the regions deleted from mutant chromosomes. The *gumB* through *M* genes (GenBank accession U22511) span about 14 kbp and the *spsG* through *urf34* genes (GenBank accession
10 U51197) include about 29 kbp.

The original cosmid clones and specific subcloned segments (diagramed in Figure 2) were transferred by conjugal mating into *X. campestris* and *Sphingomonas* recipients. The type of EPS secreted into the medium was determined from the appearance of colonies and liquid cultures, and from the physical properties and carbohydrate
15 compositions of the recovered polysaccharides.

As described in Table 1 and the legend, *X. campestris* X59 (Gum⁺), *Sphingomonas* S88 (Sps⁺) and the polysaccharide-negative mutant S88m265 (Sps⁻), have readily distinguished colony morphologies and characteristics in liquid culture. From a visual inspection, one can not only determine if any EPS is being secreted, but also
20 whether the EPS is sphingane S-88 or xanthan gum. EPS samples were acid hydrolyzed, and the identity and amounts of monosaccharide(s) were determined. The xanthan gum secreted from *X. campestris* contained two neutral sugars, with glucose representing about 67% and mannose about 33% of the sum of the peak areas for the neutral sugars on the HPLC chromatograms. By contrast, sphingane S-88 contained about 18% rhamnose, 59%
25 glucose, and 23% mannose. The sugar components distinguished xanthan gum from sphingane S-88.

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The colonial appearance (Sps^+) and composition of neutral sugars from the polysaccharides secreted by the recipient S88m265 carrying plasmid S88c1 indicated that the plasmid, which carries a normal *spsB* gene, restored sphingan S-88 synthesis to the mutant. Plasmid XC1483 with the *X. campestris gumD* gene also restored sphingan S-88
5 synthesis to S88m265.

Of particular interest is that a mixture of neutral sugars composed of about one-fourth sphingan S-88 and three-fourths xanthan gum was obtained when plasmid XCc8, which carries *gum* genes, was introduced into S88m265. This recombinant strain has all of the genes needed to make both exopolysaccharides in strain S-88. Thus, with
10 the present invention, it is possible to obtain two different exopolysaccharides from the same organism at the same time.

TABLE 1. Sugar compositions of bacterial exopolysaccharides

Donor plasmids	Bacterial recipient and phenotype	Recombinant phenotype ^a	Percent of total neutral sugars		
			Rha	Glc	Man
—	<i>X. campestris</i> X59 Gum ⁺		0	67	33
—	<i>Sphingomonas</i> S88 Sps ⁺		18	59	23
S88c1	S88m265 (SpsB ⁻)	Sps ⁺	19	62	19
XC1483	"	Sps ⁺	22	61	17
XCc8	"	Sps/Gum ⁺	7	64	29
S88c3	S88ΔTn358(Sps ⁻)	Sps ⁺	19	63	19
XCc8	"	Gum ⁺	0	64	36
XC600	"	Gum ⁺	0	62	37
XC566	S88ΔTm372(Sps ⁻)	Gum ⁻			
XCc8	S88m134(SpsB ⁻ RhsD ⁻)	Gum ⁺			
XCc8	<i>S. paucimobilis</i> ATCC 29837	Gum ⁺			

^a Gum⁺ indicates a wild-type *X. campestris*-like appearance caused by the secretion of viscous xanthan gum, with large (3-5 mm in four days at 30°C), shiny, mucoid, light-yellow-colored colonies on solid YM medium and a viscous culture broth in liquid YM medium with non-aggregated cells. Sps⁺ indicates a wild-type appearance typical of *Sphingomonas* strains secreting a capsular sphingon polymer: colonies are opaque to transmitted light, shiny but not viscous, and produce viscous liquid culture broths containing aggregates of cells.

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By deleting certain *sps* genes from the S88 chromosome we obtained synthesis in *Sphingomonas* of xanthan gum alone. Although plasmid S88c3 (*sps* genes) restored synthesis of sphingan S-88 to the deletion mutant S88 Δ Tn358, plasmids XCc8 and XC600 (*gum* genes) caused the synthesis of a polysaccharide that matched the neutral
5 sugar percentages of xanthan, and lacked rhamnose.

Plasmid XCc8 caused the synthesis of only xanthan gum in a double mutant of *Sphingomonas* (S88m134) which has defects in glucosyl-IP transferase (SpsB⁻) and in synthesis of the essential dTDP-rhamnose substrate (RfbD⁻). We also observed xanthan gum synthesis in the type strain for the *S. paucimobilis* genus, ATCC 29837, which
10 otherwise does not secrete any polysaccharide. Physical studies on this polysaccharide detailed in Example 3 confirmed that it was xanthan gum.

Example 2

Detection of gene function in recombinants

In order to determine if the acetylase (*gumF* and *G*) and pyruvylase (*gumL*)
15 genes of *X. campestris* were functioning in *Sphingomonas* we measured the amounts of each component for samples of the recombinant and commercial xanthan gums. The degree of acetylation for the recombinant sample (S88 Δ Tn358 with plasmid XCc8) exceeded that for the commercial xanthan gum by a few percent and was similar to the degree of acetylation for xanthan gum made by *X. campestris* X59 while growing under the
20 same conditions as the recombinant *Sphingomonas*. The recombinant samples were 4-6% by weight as pyruvate compared to 5-6% for commercial xanthan (Keltrol) and xanthan made by *X. campestris* X59.

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Example 3Physical analyses of recombinant xanthan gum

Three physical properties of recombinant and commercial xanthan gum were compared. First, the viscosity synergism expected for mixtures of xanthan and guar gums was observed for the recombinant samples (Table 2). Solution viscosities were measured for samples with and without added guar gum. The viscosities of the mixtures of guar gum with either commercial xanthan gum or the recombinant samples were higher than the sum of the viscosities of the unmixed polysaccharides. Second, xanthan gum is unique in forming a rigid gel in the presence of locust bean gum. Rigid gels were formed by mixing locust bean gum with commercial xanthan gum or with any one of three recombinant samples: plasmid XCc8 in either S88m265, S88ΔTn358, or *S. paucimobilis* ATCC 29837. Third, the viscosity of each recombinant xanthan sample was shear thinning like commercial xanthan gum. These three physical tests confirmed that the EPS secreted by the recombinant *Sphingomonas* strains was comparable to xanthan gum.

TABLE 2. Viscosity synergism for mixtures of exopolysaccharides and guar gum

EPS	Viscosity (cp) ^a	
	EPS alone	EPS with guar
None	—	4
xanthan gum	22	49
X59	27	63
S88m265/XCc8	7	18
S88ΔTn358/XCc8	9	27
ATCC29837/XCc8	7	29

^a Centipoise (cp) for spindle 18 at 12 rpm for final concentrations of each polymer at 0.1% in 100 mM KCl at room temperature

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Example 4Alternative culture conditions

The results in Table 3 indicate that the recombinant *Sphingomonas* strains, in contrast to *X. campestris*, converted either lactose or glucose to xanthan gum to a similar extent.

TABLE 3. Cell densities and xanthan gum yields for shake flask cultures.

10	Growth medium, temperature, and sugar substrate	X59	S88ΔTn358 with XCc8	ATCC 29837 with XCc8
		A600 mg	A600 mg	A600 mg
15	1/4 YM-G			
	30°C glucose	1.1 43	6.0 62	2.9 37
	33°C glucose	0.8 34	4.8 49	2.6 33
	30°C lactose	0.4 16	5.4 67	3.4 39
20	M9+YE			
	30°C glucose	2.4 84	2.1 30	4.9 55
	30°C lactose	0.2 9	2.3 30	6.3 53

1/4 YM-G and M9+YE were supplemented with either glucose or lactose to 2% w/v. Culture density was measured as the absorbance at 600 nm. The yield of xanthan gum (mg) is the average for samples of 10 ml taken from two separately inoculated flasks after 48 h (1/4YM-G) or 42 h (M9+YE). The cultures were centrifuged to remove cells before precipitation of the polysaccharides with alcohol.

Production rates and yields for large scale xanthan gum fermentations are sensitive to temperature and aeration. The highly viscous broth requires considerable stirring and cooling to achieve maximum productivity. Although *X. campestris* produces xanthan gum optimally at about 28°C, *Sphingomonas* strains are known to grow at temperatures up to about 37°C.

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As shown in Table 3, the recombinant *Sphingomonas* strains grew at 30°C and 33°C. In the case of recombinant ATCC 29833 with XcC8, the yields of gum were about equivalent to the native gum producer X-59 at both 30°C and 33°C in ¼YM-G media. However, the case of the recombinant S88ΔTn358 with XCc8 in ¼YM-G media, the yields of gum were significantly above those of the native gum producer X59 at both temperatures.

This is an important aspect of the present invention since, as the fermentation is exothermic, a major energy requirement is cooling of the fermentation broth. With the present invention, the fermentation can be carried out at a higher temperature, in the range from about 30 to 33 °C. This means that less cooling is required and a substantial energy cost savings can be realized with the present invention as compared with the conventional fermentation conditions used for xanthan gum production.

Example 5

Reduction of cellulase contamination in xanthan gum

The presence of contaminating cellulase in xanthan gum is disadvantageous in commercial applications where xanthan is mixed with or contacts cellulosic polymers. As judged by measuring the zones of hydrolysis surrounding cultures spotted onto agar plates containing carboxymethylcellulose, we found that the inventive *Sphingomonas* recombinants showed less than one-eighth of the cellulase activity observed for *X. campestris* strain X59. This means that xanthan gum as produced from the inventive strains contains significantly decreased amounts of contaminating cellulase as compared

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with xanthan gum obtained from X59. As used herein, "as produced" means the xanthan product as obtained directly from the broth without any steps, after-treatments or procedures taken to remove cellulase therefrom. Accordingly, the as produced xanthan gum obtained with the present invention is advantageous since it avoids the increased costs and steps normally required with conventional xanthan product to remove or decrease the cellulase content.

Example 6

Prophetic Example To Show How to Construct and Use Subsets of the Larger Segment

Construction and use of foreign genetic material from the genome of *Xanthomonas* lacking the acetylase (*gumF* and *gumG*), pyruvylase (*gumL*), or glycosyl transferase (*gumD*) genes.

Three segments of DNA containing the *gumBCDE*, *gumHIJK*, and *gumM* genes can be isolated from the recombinant plasmid carrying the XC6000 portion of the *Xanthomonas* genome. Similarly, two segments containing the *gumBC* and *gumEFGHIJKLM* genes can be isolated. The genes can be prepared by cleavage of the XC600 segment with specific restriction endonucleases or by amplification using the polymerase chain reaction, and then purified by electrophoresis through an agarose gel. When the polymerase chain reaction is used the primer segments will contain specific sequences for restriction endonucleases. The three segments will be assembled by DNA ligation as a contiguous set of genes as in a genetic operon and inserted by DNA ligation

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into a plasmid vector that can be introduced into *Sphingomonas*. The DNA sequence of the *gum* region (Genbank number U22511, seq. ID No. 1) provides the positions of the individual *gum* genes, the available restriction endonuclease sites, and the sequences required to synthesize specific primers for polymerase chain reaction. All of the above-

5 described recombinant DNA methods are routine for one of average skill in this art. The plasmid can be one of several broad host range mobilizable vectors, such as pRK311. Alternatively, the foreign genes can be inserted into the *Sphingomonas* chromosome by using a vector plasmid which cannot replicate autonomously with *Sphingomonas*.

Alternative DNA sequences can be inserted into the assembled segment to modify or

10 stimulate gene expression, such as, gene regulation sequences, promoter sequences for RNA polymerase, or ribosome binding sequences. By using modifications of these methods, one can assemble different segments lacking any one of or a combination of the *gumF*, *gumG*, *glumL*, or *gumD* genes, and thereby produce xanthan gum lacking acetyl and/or pyruvyl side groups, or lacking the glycosyl transferase GumD, where a foreign

15 glycosyl transferase gene substitutes for the GumD function.

Alternatively, one can inactivate any one of or any combination of the *gumF*, *gumG*, *gumL* or *gumD* genes by site-specific mutagenesis to alter the specific amino acid sequence for the proteins. Available methods for this mutagenesis include chemical changes to the DNA sequence or insertions of foreign DNA such as insertion sequences or

20 transposons.

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It is to be understood that the foregoing examples are exemplary and explanatory only and are not restrictive of the invention. Various changes may be made to the embodiments described above by one of skill in the art without departing from the scope of the invention, as defined by the following claims.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>10</u> , line s <u>5-10</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐	
Name of depositary institution <u>American Type Culture Collection</u>	
Address of depositary institution (including postal code and country) <u>10801 University Blvd.</u> <u>Manassas, VA 20110-2209 (USA)</u> <u>(previously at 12301 Parklawn Drive, Rockville, MD 20852)</u>	
Date of deposit <u>27 June 1997</u>	Accession Number <u>ATCC 98479 / ATCC 98480</u>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet ☐	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only ☒ This sheet was received with the international application Focal point specialist International Operations Authorized officer <u>47000 10-5747</u> 12 JUN 1998	For International Bureau use only ☐ This sheet was received by the International Bureau on: Authorized officer
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We claim:

1. A method of increasing the yield of a non-native bacterial polysaccharide produced in a recombinant bacterial host, the method comprising inactivating a native polysaccharide production in the bacterial host.
2. The method of claim 1, wherein the step of inactivating the native polysaccharide production comprises inactivating a glycosyl transferase.
3. The method of claim 1, wherein the step of inactivating the native polysaccharide production comprises the deletion of one or more native genes.
4. The method of claim 1, wherein the step of inactivating the native polysaccharide production comprises the mutation of one or more native genes.
5. A method of producing a bacterial polysaccharide from a sugar source which the polysaccharide's native bacterial producer is incapable of metabolizing, comprising transforming a bacterial host with a portion of the genome of the polysaccharide's native bacterial producer, said bacterial host being capable of transforming the sugar source into a sugar which can enter the synthesis pathway of the bacterial polysaccharide, and said genome portion comprising those genes which are necessary for establishing the production of the polysaccharide in the bacterial host.
6. The method of claim 5, further comprising inactivating a native polysaccharide production in the bacterial host.
7. The method of claim 6, wherein the step of inactivating the native polysaccharide production in the bacterial host comprises inactivating a glycosyl transferase.

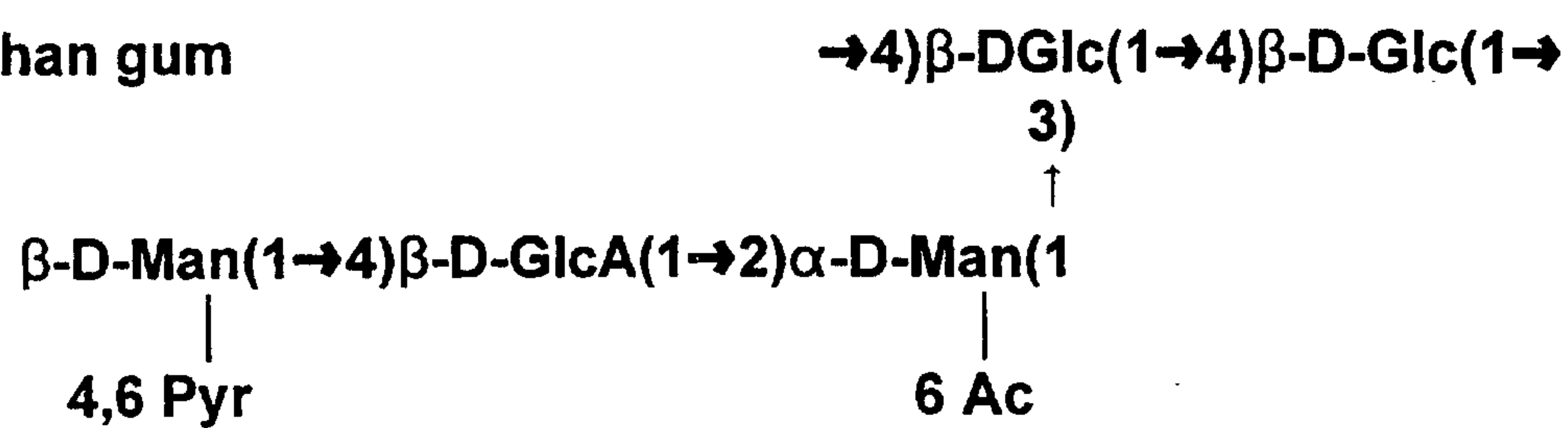
8. A method for making a recombinant xanthan producing *Sphingomonas* species strain, comprising transferring a portion of the genome of *Xanthomonas campestris* into a *Sphingomonas* species, the genome portion comprising a set of genes selected from the group consisting of the *gumB*, *gumC*, *gumD*, *gumE*, *gumF*, *gumG*, *gumH*, *gumI*, *gumJ*, *gumK*, *gumL* and *gumM* genes of the *X. campestris* genome, wherein the transfer of the set of genes enables recombinant xanthan production in the *Sphingomonas* species.
9. The method of claim 8, wherein the genome portion comprises the *gumB*, *gumC*, *gumD*, *gumE*, *gumF*, *gumG*, *gumH*, *gumI*, *gumJ*, *gumK*, *gumL* and *gumM* genes.
10. The method of claim 8 wherein the set of genes excludes at least one of the genes in the group consisting of *gumD*, *gumF*, *gumG* and *gumL*.
11. The method of claim 8, wherein the genome portion is on a plasmid.
12. The method of claim 8, wherein the genome portion is integrated into the *Sphingomonas* species' genome.
13. The method of claim 8, further comprising inactivation of native sphingan production in the *Sphingomonas* strain by deletion or mutation of one or more of the *Sphingomonas* strain's *sps* genes.
14. A recombinant *Sphingomonas* species strain which is transformed with a set of genes selected from the group consisting of the *gumB*, *gumC*, *gumD*, *gumE*, *gumF*, *gumG*, *gumH*, *gumI*, *gumJ*, *gumK*, *gumL* and *gumM* genes of the *X. campestris* genome and produces and secretes xanthan gum into media in which the recombinant *Sphingomonas* species is grown.
15. The method of claim 14 wherein the set of genes excludes at least one of the genes in the group consisting of *gumD*, *gumF*, *gumG* and *gumL*.

16. The recombinant strain of claim 14, wherein the set of genes is on a replicating plasmid in the *Sphingomonas* strain's cells.
17. The recombinant strain of claim 14, wherein the set of genes is integrated into the *Sphingomonas* strain's genome.
18. A method of producing xanthan gum from lactose, comprising incubating a recombinant *Sphingomonas* species strain transformed with a set of genes selected from the group consisting of the *gumB*, *gumC*, *gumD*, *gumE*, *gumF*, *gumG*, *gumH*, *gumI*, *gumJ*, *gumK*, *gumL*, and *gumM* genes of a *X. campestris* genome in a media containing lactose and harvesting the xanthan gum from the media.
19. The method of claim 18 wherein the set of genes excludes at least one of the genes in the group consisting of *gumD*, *gumF*, *gumG* and *gumL*.
20. The method of claim 18, wherein the lactose of the media is provided as a whey waste byproduct of cheese production.
21. A method for making xanthan gum comprising subjecting a recombinant *Sphingomonas* species strain transformed with a gene required for the production of xanthan gum in the *Sphingomonas* species from the genome of *X. campestris* to submerged aerobic fermentation conditions and harvesting the xanthan gum from the fermentation.
22. Xanthan gum as produced by the method of claim 21 and having substantially reduced cellulase activity relative to xanthan gum produced from strain X59 by submerged aerobic fermentation and harvesting from the fermentation.
23. The xanthan gum of claim 22 which has at least 50 percent less cellulase activity relative to xanthan gum produced from strain X59.

24. The method of claim 21 wherein the fermentation is carried out a temperature in the range from about 30 to 33° C.
25. Strain ATCC 98479.
26. Strain ATCC 98480.
27. A method of producing at least two exopolysaccharides in a recombinant bacterial host, comprising transforming a bacterial host with a portion of the genome of a non-native polysaccharide's native bacterial producer, said genome portion comprising those genes which are necessary for establishing the production of the polysaccharide in the bacterial host, growing the transformed bacterial host and harvesting the non-native polysaccharide and at least one polysaccharide native to the bacterial host.

FIGURE 1

Xanthan gum



Sphingan S-88

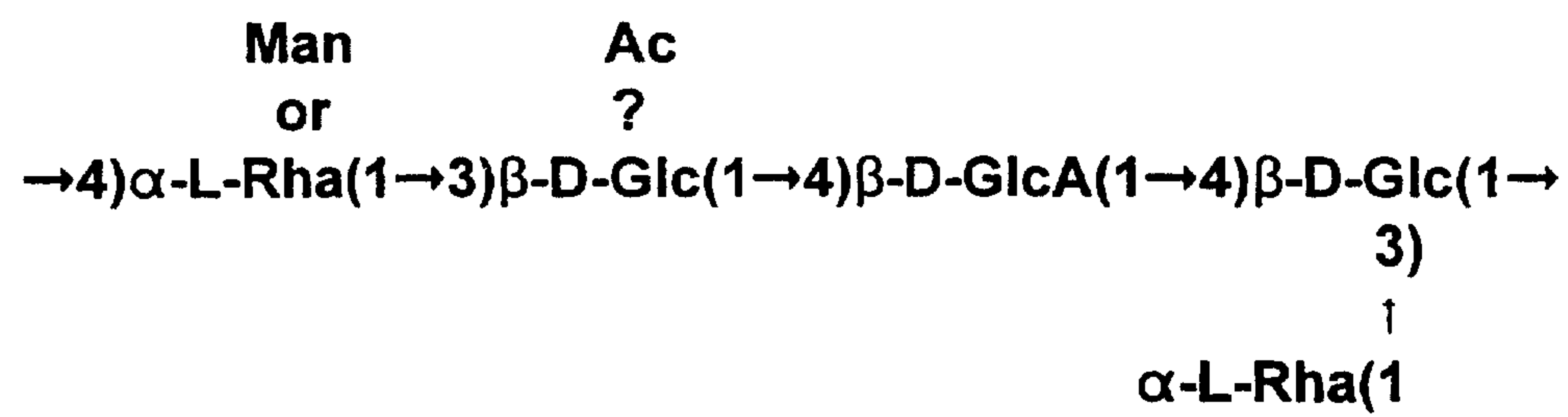
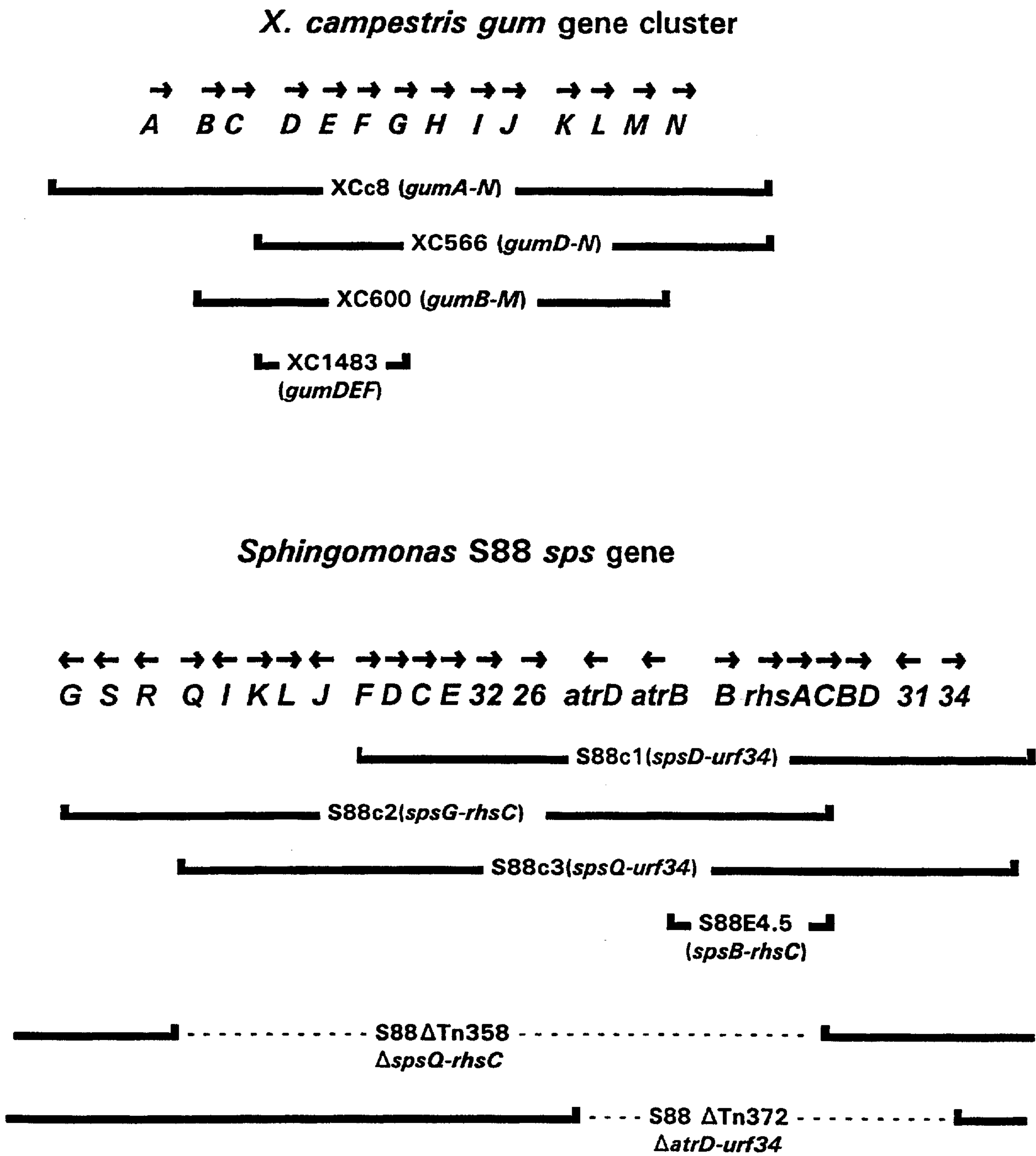


FIGURE 2



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SEQUENCE LISTING

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 Mikolajczak, Marcia
 Yamazaki, Motohide
 Thorne, Linda
 Armentrout, Richard W

<120> Production of Xanthan Gum by Sphingomonas Bacteria
 Carrying Genes from Xanthomonas Campestris

<130> seq list for appl filed from pro. appl

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