

## Description

## BACKGROUND OF THE INVENTION

5 Xanthan gum is produced naturally by bacteria of the genus Xanthomonas, in particular by microorganisms of the species X. campestris. Xanthan gum is widely used in a variety of applications due to its unusual physical properties, i.e., its extremely high specific viscosity and its pseudoplasticity. In two specific applications, xanthan gum is used in foods as a thickening agent and in enhanced oil recovery as a mobility control and profile modification agent. In addition, xanthan gum is useful in the formulation of petroleum  
10 drilling fluids.

Chemically, xanthan gum is an anionic heteropolysaccharide. The repeating unit of the polymer is a pentamer composed of five sugar moieties, specifically two glucose, one glucuronic acid, and two mannose moieties. The sugar residues are arranged such that the glucose moieties form the backbone of the polymer chain, with side chains of mannose-glucuronic acid-mannose residues generally extending from  
15 alternate glucose moieties. Usually, this basic structure is specifically acetylated and pyruvylated as described, for example, by Janson, E.P. et al., in Carbohydrate Research 45:275-282 (1975), specifically incorporated herein by reference.

To date, Xanthomonas campestris and related Xanthomonas species have been the sole source available for the production of xanthan gum. However, these organisms have low temperature optima (27° -  
20 30° C), slow growth rates and are obligate aerobes, all of which increase the cost of fermentation. Xanthan production drastically increases the viscosity of the fermentation broth, thus reducing the oxygen transfer rate, and necessitating the use of expensive aeration, cooling and agitation equipment to achieve desired product concentrations.

The present inventors have discovered portable DNA sequences encoding a gene cluster required for  
25 xanthan production and have cloned, on plasmid vectors, these portable sequences. When used in an appropriate host, particularly a denitrifying bacterium, these plasmid vectors will cause the production of xanthan gum according to the method of the present invention in an economical and commercially feasible manner. This technology could also be employed to produce variants of xanthan gum. Such variant  
30 polysaccharides are known to be produced by mutant strains of X. campestris that contain mutations within the chromosomal copy of the gene cluster responsible for xanthan production. Examples of these variant gums are disclosed in United States Patent Application Serial No. 762,878 of Vanderslice et al. entitled "A Polysaccharide Polymer Made by Xanthomonas," filed August 6, 1985 and United States Patent Application  
35 Serial No. 844,435 of Doherty et al. entitled "Family of Xanthan-Based Polysaccharide Polymers Including Non-Acetylated and/or Non-Pyruvylated Gum and Acetylated and Non-Acetylated Polytetramer Gum," filed March 26, 1986. Both of these patent applications are specifically incorporated herein by reference. Cloning a portable DNA sequence that contains such a mutation onto a plasmid vector and subsequent transfer of that recombinant plasmid into an appropriate bacterium will result in synthesis by that bacterium of a polysaccharide equivalent to the particular xanthan gum variant polysaccharide produced by the mutated X. campestris strain carrying that mutation in its chromosome.

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## SUMMARY OF THE INVENTION

One object of the present invention is to provide a method for the production of xanthan gum in which the fermentation may be conducted at a temperature greater than 30° C and/or which may be conducted  
45 under anaerobic conditions. An additional object of the present invention is to provide a recombinant-DNA mediated method for the production of xanthan gum using microorganisms which are capable of polysaccharide production and which are preferably capable of growth at or above 30° C and/or under anaerobic conditions. The xanthan gums produced by this method are chemically equivalent to that produced by Xanthomonas campestris. Additional polysaccharides, created by mutations in various biosynthetic genes,  
50 can also be produced in alternative hosts.

To facilitate the recombinant-DNA mediated synthesis of these polysaccharides, it is a further object of the present invention to provide portable DNA sequences capable of directing production of polysaccharides. It is also an object of the present invention to provide vectors containing these portable sequences. These vectors are capable of being used in recombinant systems to provide commercially  
55 useful quantities of xanthan gums and other polysaccharides.

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 be learned from practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and

combinations 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 xanthan gum are set forth, which methods utilize microorganisms capable of polysaccharide production, preferably at or above 30 °C and/or under anaerobic conditions. The polysaccharides produced by these methods are in one embodiment chemically equivalent to those produced by Xanthomonas campestris and in another embodiment are chemically equivalent to the variant gums disclosed by Vanderslice et al., supra, and Doherty et al., supra.

The portable sequences may be either synthetic sequences or restriction fragments ("natural" DNA sequences). In a preferred embodiment, a portable DNA sequence is isolated from a X. campestris library and is capable, when transferred into an alternative host, of directing the production of a xanthan gum which is chemically equivalent to that produced by Xanthomonas campestris.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA method is disclosed which results in microbial manufacture of xanthan gum and other polysaccharides using the portable DNA sequences referred to above. This recombinant DNA method comprises:

- a) preparation of a DNA sequence as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in E. coli LE392) or variants thereof capable of directing an non-Xanthomonas host to produce a xanthan polysaccharide or a xanthan variant polysaccharide structurally related to xanthan;
- b) cloning the DNA sequence into at least one vector capable of being transferred into and replicating in a host microorganism, such vector containing elements for the expression of the biosynthetic enzymes encoded by the DNA sequence;
- c) transferring the vector containing the DNA sequence into a host microorganism capable of producing polysaccharide under the direction of the DNA sequence;
- d) culturing the host microorganism under conditions appropriate for maintenance of the vector and synthesis of the polysaccharide; and
- e) harvesting the polysaccharide.

In a preferred embodiment of the present invention, the portable DNA sequence is comprised of DNA sequences capable of directing production of the following enzymes: Transferase I; Transferase II; Transferase III; Transferase IV; Transferase V; Acetylase; Ketalase; and Polymerase. These enzymes, which are used in xanthan gum biosynthesis, are depicted in Figure 1 and described more fully below.

To further accomplish the objects and in further accord with the purposes of the present invention, a series of cloning vectors are provided, each of which contains at least one of the portable DNA sequences discussed above. In particular, plasmids pRK290-H336 and pX209 are disclosed.

Strains E. coli LE392(TX209), bearing plasmid pX209, and strain E. coli LE392(pRK290-H336), bearing plasmid pRK290-H336, have been deposited in the American Type Culture Collection, Rockville, Maryland, on March 21, 1986 under Accession Nos. 67051 and 67049, respectively.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate various embodiments of the invention and, together with the description, serve to explain the principles of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the biosynthetic pathway of xanthan gum synthesis in X. campestris.

Figure 2 is a restriction map of lambda 655(+).

Figure 3 is a restriction map of lambda 708(+).

Figure 4 is a BamHI restriction map of the region of the X. campestris genome containing the gum gene cluster. The numbers are the molecular size of the fragment in kilobases (kb).

Figure 5a shows the general structure and pertinent restriction endonuclease cleavage sites of cloning vector pMW79.

Figure 5b depicts a representative, but not inclusive, sample of segments of gum gene DNA cloned into the BamHI site of pMW79 from either partial or complete digests of various lambda recombinants with BamHI as described in Example 6.

Figure 6 depicts partial restriction maps of two recombinant lambda phages (lambda 708(8) and lambda 655(B)) shown in relation to the BamHI restriction map of the X. campestris DNA in the vicinity of the gum gene cluster. The cloned segments of gum gene DNA carried in four recombinant plasmids (pX206, pX207, pX208, and pX209) are also shown. For simplicity, the pMW79 vector sequences of these plasmids are not depicted, but the details of these constructions are given in Example 6.

Figure 7 shows the positions, within the BamHI restriction map of the gum gene cluster DNA, of 22 insertion mutations. Open circles indicate in vitro-generated insertions of the BglIII Tet<sup>r</sup> fragment of Tn10, while filled circles represent in vivo-derived Tn10 insertions. Phenotypic classification of mutants carrying these insertions, as described in Example 2, is shown below.

5 Figure 8 depicts the location within the BamHI restriction map of a set of six representative mutations in the gum gene cluster. Strains X925, X928, X975, X1008, and X655 carry insertion mutations of Tn10 or the BglIII Tet<sup>r</sup> fragment of Tn10 at the positions indicated. Strain X974 carries a deletion of the 1.35 kb BamHI fragment and a substitution of the BglIII Tet<sup>r</sup> fragment at that position. Below the restriction map are shown representative plasmids used in complementation experiments with the above mutants. For simplicity, only  
10 cloned X. campestris sequences are shown; the vector pMW79 sequences are omitted. A plus sign (+) indicates successful complementation of the mutant by the plasmid, whereas a minus sign (-) denotes failure to complement. Details of the experiments and interpretations are given in Example 8.

Figure 9 shows a partial restriction map of plasmid pRK290 and various segments of DNA cloned out of the recombinant lambda phage 655 (L') and into the BglIII site of pRK290 as detailed in Example 10.

15 Figure 10 consists of the nucleotide sequence of a 16,080 base pair segment of Xanthomonas campestris DNA that contains a gene cluster that directs Xanthan biosynthesis.

Figure 11 shows an overview of the organization and structure of the genes contained in the 16 kb segment of DNA. The top line in the figure is a BamHI restriction map and indicates the location of each of the BamHI restriction sites in the sequence. The line drawn above the frame analysis curves shows the  
20 approximate position of some of the mutations that have been isolated and characterized. The frame analysis curves presented show the distribution of G + C content at the first (blue line), second (red line), and third (black line) nucleotide positions. Note that the distribution of G + C content at the three nucleotide positions is non-random throughout the entire sequence, indicating that virtually all of this DNA codes for protein products. The reading frame of each protein is defined by the nucleotide position having an  
25 intermediate value within each region of non-random G + C distribution. The points where the G + C distribution at the three nucleotide positions change predict either the beginning or end of a gene or the end of one gene and the beginning of the next. In each case, these points were found to correlate with the presence of either a start or stop codon in the appropriate reading frame.

Below the frame analysis curves, separate arrows are drawn to indicate the location and extent of each  
30 gene in the sequence. For convenience, we designate each gene with a letter and use that letter preceded by "gp" to designate its protein product. Above each arrow, the molecular weight of the protein product is shown in kD. Below each arrow, the name of each gene product is shown as its lettered name as well as its functional name for those cases where gene function could be derived from the mutant phenotype.

Figure 13 shows potential secondary structures of putative transcription terminators identified within the  
35 DNA sequence around positions 900, 3400 and 12,400.

Figure 14 shows the folded secondary structure of the proline tRNA identified in the DNA sequence from positions 732-808.

Figure 15a shows the locations of various TnK12 insertion mutations within the cloned X. campestris DNA carried in recombinant plasmid pRK290-H336.

40 Figure 15b shows the extent of DNA deleted from the X. campestris chromosome in a series of deletion mutants. The deleted DNA is indicated by the cross-hatched box.

Figure 16a shows the locations of in vitro generated Kan<sup>r</sup> insertion and deletion mutations within plasmid pRK290-HA3.

45 Figure 16b shows the positions of seven in vitro generated Kan<sup>r</sup> insertion mutations within plasmid pRK290-H336.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors believe that biosynthesis of xanthan gum in Xanthomonas campestris proceeds  
50 via the pathway depicted in Figure 1. This figure depicts the assembly of the pentasaccharide (five-sugar) repeat unit of xanthan gum linked to an isoprenoid lipid "carrier" molecule. The assembly of this pentasaccharide is shown to proceed by sequential addition, in a specific and defined order, of the five individual sugar moieties that occur in the pentasaccharide repeat unit. Each unique sugar addition is catalyzed by a specific enzymatic activity that is specific to that particular step. The sugars are donated by  
55 specific sugar nucleotides. The enzymatic activities are referred to herein as "Transferases." The individual enzymatic activities are further designated by roman numerals I through V, denoting the step in the sequentially ordered series of sugar additions that each Transferase activity catalyzes.

The two mannose sugar moieties present in mature xanthan, and in the pentasaccharide precursor unit, are known to be modified. The mannose moiety added at step 3 by the activity of Transferase III is known to be acetylated. The fraction of this moiety present in the acetylated form in mature xanthan gum is observed to be variable. An enzymatic activity, here termed Acetylase, catalyzes the addition of the acetyl group to the mannose, although the precise point in the sequence of the biosynthetic pathway at which the Acetylase functions is currently unknown. Similarly, the mannose sugar moiety attached by the activity of Transferase V at step 5 is known to be pyruvylated. An enzymatic activity, here termed Ketalase, catalyzes the addition of the pyruvate group. Again, the fraction of these mannose moieties that are observed to be pyruvylated in mature xanthan gum is variable, and the point within the biosynthetic pathway at which the Ketalase acts is currently unknown. etc pathway at which the Ketalase acts is

Pentasaccharide precursor units are polymerized in a subsequent enzymatic reaction catalyzed by an activity referred to here as "Polymerase." The Polymerase activity catalyzes release of a pentasaccharide subunit from its attached isoprenoid lipid carrier molecule and concerted attachment of that pentasaccharide unit onto a lipid-linked pentasaccharide, decasaccharide, or higher order array of the basic pentasaccharide repeat with the resulting increase in the degree of polymerization of recipient nascent xanthan molecules.

As noted in the copending United States Patent Applications of Vanderslice *et al.* and Doherty *et al.*, *supra*, both of which are specifically incorporated herein by reference, other polysaccharides have been discovered which may be described as "variants" of xanthan. These include the "polytrimer" of Vanderslice *et al.*, both in its acetylated and non-acetylated forms, as well as the "polytetramer," both acetylated and nonacetylated, and the non-acetylated, non-pyruvylated, or non-acetylated and non-pyruvylated pentameric xanthan gums of Doherty *et al.* It is clear from both the foregoing description of the enzymes discovered in the xanthan pathway and from the following description of the newly-discovered mutant organisms that the materials necessary for the recombinant-DNA mediated production of these variant gums are inherently described herein. For example, polytetramer may be synthesized in a recombinant-DNA system which does not possess the DNA sequence encoding Transferase V while a non-acetylated polytetramer-synthesizing plasmid would lack both the genes encoding Transferase V and Acetylase.

A mutation of *Xanthomonas campestris* has been identified that specifically inactivated the activity of Transferase IV. A strain carrying this mutation, X655, has been described by Vanderslice *et al.* and has been deposited at American Type Culture Collection (ATCC) in Rockville, Maryland under Accession No. 53195. The identification of that specific defect in that *X. campestris* mutant strain X655 led to the cloning of *X. campestris* chromosomal DNA sequences from the region of the chromosome to which the site of the mutation in strain X655 was localized. Mutagenesis and analytical technology were used as described below in Examples 1, 2 and 7 to induce and analyze the phenotypic properties of mutations in DNA sequences near the site of the X655 mutation. As a basis for the present invention, it was believed that the genes encoding other Transferase activities would be clustered in the vicinity of the gene identified in strain X655 as encoding a necessary component of the activity of Transferase IV, because in bacteria clustering of genes for metabolic pathways is known to occur.

Knowledge of the Transferase IV defect in strain X655 was used to clone and genetically and physically analyze a large segment (35 kb) of *X. campestris* chromosomal DNA spanning the site of the mutation causing the Transferase IV defect in strain X655. Numerous mutations within that DNA were induced and characterized. Analyses of genetic locations of these mutations and the phenotypes of mutant strains carrying these mutations (Examples 2, 4 and 7) provided direct evidence that this DNA sequence in the *X. campestris* chromosome, and as a portable DNA segment on transferable plasmid vectors, contained genes encoding proteins required for the activities of Transferase I, II, III, and IV, Acetylase, and Ketalase. These data lead to the conclusion that the portable DNA segments cloned in plasmids pRK290-H336 and pX209 contain genes encoding proteins required for the activities of Transferases I through V, Acetylase, Ketalase, Polymerase, and possibly other as yet uncharacterized activities, necessary for, or related to, xanthan biosynthesis. Transfer of plasmid vectors containing this "gene cluster" for xanthan biosynthesis, as a portable DNA sequence, into bacteria other than *Xanthomonas campestris*, could thus be used to reduce the cost of production of xanthan gum by altering the conditions of the fermentation.

To date, *Xanthomonas campestris* and related *Xanthomonas* species have been the sole microbial source available for the production of xanthan gum. However, these organisms have low temperature optima (27-30 °C), slow growth rates and are obligate aerobes, all of which increase the cost of fermentation. In addition, the existing aerobic fermentation technology requires a low viscosity fermentation broth to enable the broth to have a high oxygen transfer rate. However, because xanthan is an exopolysaccharide, the xanthan production drastically increases the viscosity of the fermentation broth, thus reducing the oxygen transfer rate and necessitating the use of expensive aeration, agitation, and cooling equipment to achieve desired product concentrations.

It has been proposed by the present inventors that denitrifying bacteria, which may have higher temperature optima and faster growth rates and will have the ability to grow anaerobically if supplied with a suitable nitrogen source, could be transformed with portable DNA sequences capable of directing the production of xanthan gum. Production of xanthan in these organisms will be less expensive than in the current species which is employed. In addition, the present inventors have discovered a portable DNA sequence encoding the gene cluster responsible for xanthan production and have plasmid vectors which contain these portable sequences. When used in an appropriate host, particularly a denitrifying bacterium, these plasmids will cause the production of xanthan gum according to the method of The present invention in an economical and commercially feasible manner. In addition, bacterial hosts with only some of the proposed advantages are contemplated as alternative production hosts.

The genes responsible for conversion of xanthan precursors (UDP-glucose, UDP-glucuronic acid, GDP-mannose, acetyl-CoA, and phosphoenolpyruvate) to xanthan are clustered in the *X. campestris* genome and have been cloned. In the present invention, these genes will be introduced into suitable alternative hosts, and gum biosynthesis measured. Corrective steps are outlined in the eventuality that the alternative hosts, with the biosynthetic cluster present, do not synthesize xanthan gum.

Additional embodiments of the present invention are envisioned as employing other known or currently undiscovered vectors which would contain one or more of the portable DNA sequences described herein. In particular, it is preferred that these vectors have some or all of the following characteristics: (1) be stable in the desired host; (2) be capable of being present in an appropriate copy number in the desired host; (3) possess a regulatable promoter; and (4) have at least one DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the portable DNA sequence will be inserted.

The following, non-inclusive list of bacterial hosts and plasmid vectors is believed to set forth combinations which can easily be altered to meet the above-criteria and are therefore preferred for use in the present invention (Table 1). Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teachings contained herein.

Table I

| HOST VECTORS   | COMMENTS |                                          |
|----------------|----------|------------------------------------------|
| PSEUDOMONAS    | RSF1010  | Some vectors useful in broad             |
| P. AERUGINOSA  | Rms149   | host range of Gram-negative              |
| P. PUTIDA      | pKT209   | bacteria including Xanthomonas           |
|                | RK2      | and Agrobacterium.                       |
|                | pSa727   |                                          |
| CLOSTRIDIUM    | pJU12    | Shuttle plasmids for <i>E. coli</i> and  |
| C. PERFRINGENS | pJU7     | <i>C. perfringens</i> construction, ref. |
|                | pJU10    | Squires, C. <u>et al.</u> (1984) Journal |
|                | pJU16    | Bacteriol. 159:465-471.                  |
|                | pJU13    |                                          |

The large cluster of gum biosynthetic genes has been cloned on a plasmid containing a broad host range origin of DNA replication that can be transferred and maintained in a wide variety of gram-negative bacterial species, including *X. campestris* and *X. campestris* strain X1107, which contains a deletion of all the known gum biosynthetic genes. Plasmid pRK290-H336 converts X1107 into a xanthan producing strain through complementation.

When a plasmid such as pRK290-H336 is placed in an alternative host, many steps must occur properly in order for xanthan biosynthesis to occur. First, the genes encoding the biosynthetic enzymes must be transcribed and translated to give functional biosynthetic enzymes at an appropriate level. Second,

the alternative hosts must contain sufficient biosynthetic capacities for acetyl-CoA, phosphoenolpyruvate, UDP-glucose, UDP-glucuronic acid, and GDP-mannose so that the biosynthetic enzymes can polymerize those precursors into xanthan gum. Third, the biosynthetic enzymes encoded by the cluster must aggregate if such a multi-protein complex is the operative biosynthetic unit. Fourth, the architecture of that complex (or the individual enzymes) must provide vectorial polysaccharide biosynthesis so that the xanthan will be secreted into the culture medium.

The practice of genetics, molecular biology, biochemistry, fermentation engineering, microbial physiology, and recombinant DNA technology, by one skilled in the art, makes likely the straightforward and obvious isolation of alternative hosts that express the xanthan biosynthetic genes and that can produce extracellular xanthan gum.

## EXAMPLES

### Example 1

This example shows the methods of mutagenesis and screening employed to generate the mutant strains which are xanthan gum deficient.

B1459 S4-L was obtained from the Northern Regional Research Laboratories of the U.S. Department of Agriculture. It was genetically marked with a chromosomal resistance to streptomycin and was used as a recipient in a conjugation with *E. Coli* LE392 containing plasmid pRK2013::Tn10. Plasmid pRK2013 contains Tn903 which encodes kanamycin resistance as described by Figurski, D.H., and Helinski, D.R. in Proc. Natl. Acad. Sci., U.S.A., 76: 1648-1652 (1979), specifically incorporated herein by reference. The plasmid cannot replicate in *X. campestris*. Transposon Tn10 encodes resistance to tetracycline. Transconjugants were selected which were resistant to streptomycin and kanamycin, or streptomycin and tetracycline. The former occurred at a frequency of about  $4 \times 10^{-6}$  per recipient and presumably resulted from a transposition of Tn903. The latter occurred at a frequency of about  $3 \times 10^{-6}$  per recipient and presumably resulted from a transposition of Tn10 into the genome of *Xanthomonas campestris*.

Auxotrophs were found among these transconjugants at a frequency of about 2%; their needs were widely distributed among the various nutritional requirements. This indicates that these transposons do not have a particularly preferred locus for insertion in *X. campestris*. Prototrophic revertants of the auxotrophs were selected, and most were found to be drug-sensitive; this suggests that the auxotrophies were caused by transposon insertion.

To screen for xanthan gum deficient mutants among the doubly resistant transconjugants, Congo Red dye (200 ug/ml), which enhances the morphological distinction between xanthan gum producing and non-producing colonies, was added to the solid media. Colonial morphology was examined after 7 to 12 days incubation at 30°C. Xanthan gum deficient mutants were found at a frequency of approximately  $10^{-4}$ . Henceforth, strains that fail to make xanthan *in vivo* are termed Gum<sup>-</sup> strains, and those caused by insertion of Tn10, also Gum<sup>-</sup> strains, may be additionally designated as gum::Tn10 mutants.

### Example 2

This example demonstrates the biochemical phenotypes of the Gum<sup>-</sup> mutant strains and the methods used to assess the phenotypes.

The basic method relating to the use of a cell-free system to study the biosynthetic pathway of xanthan gum is described by Ielpi, L., Couso, R.O., and Dankert, M.A. in FEBS Letters 130: 253-256 (1981), specifically incorporated herein by reference. It has been found that a modified version of this method may be employed to analyze the Gum<sup>-</sup> isolates described herein. For this novel method, the *in vitro* cell-free system is prepared generally by lysing cells of a microorganism, preferably *Xanthomonas campestris*, in the presence of a suitable buffer, preferably with EDTA, and obtaining the appropriate biosynthetic enzymes which are able to subsequently process exogenously added substrates. Alternate means of lysis may be used, including but not limited to sonication, detergent treatment, enzyme treatment and combinations thereof.

Generally, to determine the defective step in the biosynthetic pathway of a Gum<sup>-</sup> mutant, a lysate of this microorganism was incubated with the appropriate substrates, which may include UDP-glucose, GDP-mannose, UDP-glucuronic acid, acetyl-CoA, and phosphoenolpyruvate. The choice of substrates is dependent on the steps which are desired to be analyzed. The biosynthetic process may, in one embodiment, be monitored by the incorporation of radiolabeled substrates into the polymeric units. Other methods that are known to those of ordinary skill in the art also may be used to allow identification of the biosynthetic

intermediates. In particular, chromatographic methods have been developed to separate and to identify the oligosaccharide intermediates after hydrolysis from the lipid carriers. These include thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

The cell-free biosynthesis of xanthan has been found to be a time-dependent, sequential process that is dependent on the addition of all three specific sugar nucleotides. The background of non-specific incorporation of labeled substrate is minimal and does not interfere with the detection of the xanthan-specific intermediates or xanthan polymer in the gum fraction.

The involvement of lipid carriers, specifically C55 isoprenoid pyrophosphate, has been shown in several polysaccharide biosynthetic pathways. Additionally, the involvement of a pyrophosphoryl-linked lipid carrier in xanthan biosynthesis has been demonstrated and confirmed. Thus, the xanthan biosynthetic intermediates, at least up to the pentasaccharide, have been found to be recoverable in the organic soluble fraction with these carrier lipids.

Using methods described herein for recovery of intermediate products, it has been discovered that, under *in vitro* conditions, mutant *X. campestris* lysates will produce accumulated intermediates and novel truncated forms of xanthan gum, even in the presence of all substrates required for normal xanthan biosynthesis. The specific blockage points indicate which particular enzyme activities are missing. Gum<sup>-</sup> strains were analyzed and assigned biochemical phenotypes. These biochemical phenotypes allow the genotypes of the mutant strains to be defined. For instance, a mutant strain that accumulates cellobiose on the lipid carrier *in vitro* is said to have a defect in the gene for Transferase III.

If the lysate of a Gum<sup>-</sup> mutant produces normal xanthan gum when supplied with all the substrates, one concludes that all of the enzymes in the biosynthetic pathway are normal. Thus, the inability to make gum *in vivo* is a result of the absence of one of the required substrates. A class of Gum<sup>-</sup> mutants which can make gum only *in vitro* when the substrates are provided was found. These Gum<sup>-</sup> mutants are discussed more fully in United States Patent Application Serial No. 843,349 of Betlach *et al.* entitled "Process for the Synthesis of Sugar Nucleotides Using Recombinant-DNA Methods," filed March 24, 1986. All of these mutants mapped away from the gum cluster.

Specific procedures for these cell-free studies are described herein. The Gum<sup>-</sup> derivatives were grown in YM (yeast-malt medium) supplemented with 2% (w/v) glucose as described by Jeanes, A., *et al.* (U.S. Department of Agriculture, ARS-NC-51, 14 pp (1976)) specifically incorporated here by reference. Cult. res were grown to late log phase at 30 °C at 300 rpm. The cells were titered on YM plus 2% (w/v) glucose plates at 30 °C. The cells were harvested by centrifugation and washed with cold Tris-HCl, 70mM, pH 8.2. Washed cells were resuspended in Tris-HCl, 70mM, pH 8.2 with 10mM EDTA and were freeze-thawed three times by a procedure similar to Garcia, R.C., *et al.* (European Journal of Biochemistry 43:93-105, (1974)) specifically incorporated herein by reference. This procedure ruptured the cells, as was evidenced by the increased viscosity of the suspensions and the complete loss of cell viability (one in 10<sup>6</sup> survivors) after this treatment. The freeze-thawed lysates were frozen in aliquots at -80 °C. Protein concentration was determined with BIO RAD assay (BIO RAD Laboratories, Richmond, California) and was found to be 5 to 7 mg cell protein per ml of lysate.

As described in Ielpi, L., Couso, R.O., and Dankert, M.A., *supra*, an aliquot of freeze-thawed lysate (equivalent to 300 to 400 ug protein), DNAase I (10 ug/ml), and MgCl<sub>2</sub> (8 mM) were preincubated at 20 °C for twenty minutes. An equal volume of 70 mM Tris-HCl, pH 8.2, with the desired radiolabeled sugar nucleotides (UDP-glucose and GDP-mannose), with or without UDP-glucuronic acid, was added and incubated at 20 °C. At various times, the reactions were stopped by the addition of EDTA to 4mM. The samples were centrifuged; the pellets were washed two times with buffer. To allow analysis of the gum fractions, the supernatants were combined, carrier xanthan (100 ug) was added, and the xanthan plus synthesized polymer were precipitated with ethanol(60%)-KCl(0.8%). The precipitated polymer was resuspended in water and reprecipitated two more times to remove unincorporated label. Radioactivity incorporated into the gum fraction was determined in a liquid scintillation counter, and the data were processed to obtain incorporation in terms of picomoles.

To allow analysis of the lipid carrier linked intermediates, the washed pellet was extracted twice with chloroform:methanol:water (1:2:0.3). The lipid-linked biosynthetic intermediates were converted to the free oligosaccharides by mild acid hydrolysis (pH 2, 90 °C, 20 min) and alkaline phosphatase treatment (bovine alkaline phosphatase, 50 mM MgCl<sub>2</sub>, and 10 mM glycine buffer pH 9.8 at 37 °C overnight). The samples were back-extracted with chloroform:methanol (2:1) and centrifuged. The aqueous phase was removed and reduced in volume in vacuo for analysis by thin-layer chromatography.

Thin-layer chromatography was carried out on silica gel (Baker 250 um, preformed lanes) with butanol:dioxane:water (35:50:20) using three developments. Compounds radiolabeled with carbon-14 were detected by autoradiography at -80 °C using Kodak X-Omat AR film with standard development. The sugar

standards were visualized with aniline diphenylamine (1.8% aniline and 1.8% diphenylamine in acidified acetone obtained from Sigma Chemical Co.). The mobility of the xanthan biosynthetic intermediates was compared to the mobility of sugar standards. For radiometric analysis of double-labeled oligosaccharides, silica gel not treated with interfering sprays was scraped, eluted, and counted with Budget-Solve Aqueous Counting Cocktail (RPI) in plastic vials in a Beckman LS-7500 scintillation counter utilizing the autoquench compensation. The scintillation data were processed to obtain the absolute amounts of [<sup>3</sup>H]-labeled and [<sup>14</sup>C]-labeled materials to allow molar ratios of the sugars in the compounds to be computed.

The Gum<sup>-</sup> strains analyzed *in vitro* were assayed in several ways: (1) radiolabeled UDP-glucose alone to assess the charging of the carrier lipid with glucose or cellobiose, (2) unlabeled UDP-glucuronic acid and double radiolabeled UDP-glucose and GDP-mannose to determine the molar ratio of glucose and mannose, and (3) unlabeled UDP-glucose and double radiolabeled GDP-mannose and UDP-glucuronic acid to compare the molar ratio of mannose and glucuronic acid in the intermediates and the gum fraction. Mutants suspected of defects in acetylation or pyruvylation were checked for their ability to incorporate radiolabeled acetyl-CoA and phosphoenolpyruvate by the methods of Ielpi *et al.*, *Biochem. Biophys. Res. Comm.* 102:1400-1408 (1981) and Ielpi *et al.*, *Biochem. Intern.* 6:323-333 (1983), both of which are specifically incorporated herein by reference.

The strains fell into two major phenotypes. One group was defective in gum synthesis *in vivo* and *in vitro*. All of these mutants had mutational insertions in the gum DNA cluster. The other group, although they could not synthesize polysaccharide *in vivo*, could synthesize xanthan gum *in vitro* when the substrates were provided. All of these mutants had mutational insertions in DNA unlinked to the gum cluster. These mutants were tested for the presence of the sugar nucleotides and found to be defective in various steps of the biosyntheses of sugar nucleotides.

The mutants with blocks in the gum biosynthetic pathway were found to be of several types. The possible biochemical phenotypes and our observations are presented here and in Fig. 7 where the map positions of some mutations conferring these particular phenotypes are shown.

#### Transferase I and Unknown Defects

Many mutant lysates showed poor incorporation of radiolabel in the organic fraction, with a small quantity of glucose being the only sugar detected above background. No polymeric material was detected in the gum fraction. This small quantity of glucose has been demonstrated by TLC and/or HPLC. There are several possible explanations for this phenotype. The level of glucose seen in this class is similar to the non-xanthan-specific or "unchaseable" glucose seen when all three sugar nucleotides are present for S4-L. This phenotype may be the result of a Transferase I defect, which would not allow charging of the lipid carrier with glucose. Alternatively, it could result from a defect in another gene that influences the initiation of the biosynthesis, directly or indirectly affecting the expression of Transferase I.

#### Transferase II

Two mutant lysates showing a significant accumulation of glucose on the lipid carrier were observed. The glucose was not polymerized to cellobiose on the lipid carrier. These lysates were unaffected by the presence of GDP-mannose and UDP-glucuronic acid. No radiolabeled material was found in the gum fraction. This defect is thought to be in the gene for Transferase II.

#### Transferase III

The radiometric analysis of the cell-free biosynthetic reaction mixes from some lysates showed that, in the presence of UDP[<sup>14</sup>C]glucose, the organic fraction charges well (37% of the S4-L level). In the presence of all three sugar nucleotides, the charging with glucose was at the same high level, but there was no incorporation of either mannose or glucuronate. The gum fraction showed that no polymeric material (cellulose) was synthesized. The freed sugars from the organic fraction were analyzed by TLC and these mutants were shown to synthesize cellobiose very efficiently. The cellobiose accumulated in the organic fraction. The presence of GDP-mannose or UDP-glucuronate did not affect the accumulation of the cellobiose; the cellobiose did not appear to be processed further. These data indicate that these mutants have a defect in the Transferase III.

Transferase IV

Some mutant lysates (including lysates from X655, ATCC No. 53195) show the accumulation of the lipid-linked trimeric intermediate (described in U.S. Patent Application of Vanderslice *et al.*, *supra.*) in the organic fraction which has a molar ratio of 2:1 glucose to mannose. The gum fraction of each cell lysate in this group contains radiolabeled polytrimeric gum. These mutants are in the gene for Transferase IV, the glycosyl transferase that transfers glucuronic acid to the lipid-linked oligosaccharide precursor.

Transferase V

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Mutant strains of this type would accumulate the tetrameric oligosaccharide on the lipid carrier and presumably produce an altered polysaccharide missing the terminal mannose and pyruvate.

Polymerase

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Mutant strains with a defective Polymerase may accumulate the lipid-linked pentameric building blocks and be unable to polymerize them. This phenotype was not observed. A defective gene for the Polymerase may result in a different biochemical phenotype, such as no charging, or a lethal phenotype to the organism. Specifically, Polymerase mutants might show a Transferase I phenotype.

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Acetylase and Ketalase

These defects were found in Gum+ strains. Polysaccharide was harvested after growth by centrifugation of culture broth at 12,000 x g for 30 minutes to one hour at 10-20°C. Precipitated gum from the supernatant was analyzed after hydrolysis by HPLC. The HPLC analysis of the hydrolyzed gums show that some mutants produce xanthan gum without pyruvate, and some mutants produce xanthan gum with 2:2:1 molar ratios of glucose to mannose to glucuronate with no acetate. *In vitro* data confirmed these results. These mutations eliminate the Ketalase or the Acetylase, respectively.

30 Example 3

Example 3 is the preparation of a library of total genomic *X. campestris* DNA in lambda 1059.

Bacteriophage lambda 1059 is a substitution cloning vector constructed by Karn *et al.* in Proc. Natl. Acad. Sci. U.S.A. 77: 5172-5176 (1980), specifically incorporated herein by reference. The chromosome of this phage contains a 14 kb central region delimited by two BamHI sites. This central BamHI fragment (hereinafter referred to as the "stuffer" fragment) contains no genetic functions necessary for phage growth and can thus be removed and replaced with foreign DNA. The two arms of the vector contain all of the essential genetic functions for lambda replication and maturation. Viable phage particles are produced by ligating a DNA fragment having a size of 6 kb to 24 kb between the left and right arms of the vector DNA. Ligations of the left and right arms to each other do not yield viable phage particles because the genome size is too small for proper packaging into phage heads.

The "stuffer" fragment of lambda 1059 carries the lambda red (exo and beta genes) and gamma under the control of the leftward promoter (pL). These genes confer a Spi<sup>+</sup> phenotype on the vector, i.e., the phage is able to grow on recA<sup>-</sup> strains but is unable to grow on strains that are lysogenic for bacteriophage P2. Since pL is also located on the "stuffer" fragment, the expression of the Spi<sup>+</sup> phenotype is not affected by the orientation of the "stuffer" between the left and right arms of the vector.

Vector DNA digested with BamHI is ligated with genomic DNA prepared by digestion with any restriction enzyme that generates "sticky" ends that are compatible with the cohesive ends of BamHI (e.g., BglIII, BclI, and Sau3A). Cleavage of genomic DNA by Sau3A is an effective technique for generating a nearly random population of high molecular weight DNA fragments because the recognition site for cleavage by this enzyme occurs on an average of once in every 256 bp. Viable phage particles containing an insert of foreign DNA will express a Spi<sup>-</sup> phenotype and, thus, be able to grow on P2 lysogens but not on recA<sup>-</sup> strains.

High molecular weight (greater than 100 kb) genomic DNA was isolated from 2 liters of S4-L rif-101 using procedures described by Saito and Muria as described in Biochem. Biophys. Acta 72: 619-629, specifically incorporated herein by reference. High molecular weight *X. campestris* genomic DNA was partially digested with Sau3A using reaction conditions which generated a collection of fragments with a predominant size of 15-20 kb. In order to avoid spurious linkage from multiple ligation events, the fragments

produced by Sau3A digestion were rigorously fractionated on a 10-40% sucrose gradient to a size of 15-24 kb. The size of the DNA was confirmed by running a small aliquot on a 0.4% agarose gel.

Phage lambda DNA was isolated from phage particles purified by equilibrium centrifugation through CsCl gradients. The lambda 1059 DNA was digested with BamHI and SalI. BamHI digestion separates the left and right arms from the "stuffer" fragment. SalI digestion further cleaves the "stuffer" and thereby limits the reformation of the cloning vector during ligation. A 2 ug aliquot of the BamHI-SalI digested vector DNA was mixed with 0.6 ug of 15-24 kb fragments produced by Sau3A cleavage of X. campestris DNA and ligated with T4 ligase. The ligated DNA was packaged in vitro using lambda packaging mix obtained from Boehringer Mannheim.

Dilutions of the packaged DNA were used to infect three different E. coli strains: a nonrestrictive strain Km392, a recA<sup>-</sup> strain KRO, and a P2 lysogen strain, Q359. Strain Km392 is LE392 carrying Tn5 inserted into proC and is described by Young, R.A. in Science 222:778-782 (1983), specifically incorporated herein by reference. Infection of KM392 gave a titer of  $1 \times 10^5$ , while infection of KRO and Q359 gave titers of  $6 \times 10^4$  and  $1.2 \times 10^5$ , respectively. The titer on KM392 is a measure of the total viable phage. The titer on KRO is a measure of the number of phages without an insert of X. campestris DNA, while the titer on Q359 indicates the number of phages containing an insert of X. campestris DNA. The relatively large number of viable phage that do not contain an insert of X. campestris DNA was surprising since double digestion of the vector DNA with BamHI and SalI should have prevented the formation of a significant number of lambda 1059 particles through religation events. It should also be noted that the total number of viable phage determined by adding the titers on KRO and Q359 is approximately five-fold lower than the total viable phage determined from infection of KM392. One interpretation of these results is that the plating efficiency of phage with and without insert DNA is about five-fold less on both KRO and Q359 than on KM392. In other words, the number of phage with and without an insert of X. campestris DNA is actually five-fold greater than the combined titers of KRO and Q359 indicate.

This interpretation was tested by determining the proportion of phage growing on KM392 that contain insert DNA. The phage present in 48 isolated plaques growing on KM392 were toothpicked to drops of sterile buffer and then printed in turn on a lawn of Q359 cells, KRO cells, and KM392 cells. All of the isolates grew on KM392, 62% grew on Q359 but not on KRO, and 38% grew on KRO but not on Q359. Thus, the proportion of phage growing on KM392 that carry an insert of X. campestris DNA is 62%. This value is in good agreement with the predicted value of 66% ( $1.2 \times 10^5 / 1.8 \times 10^5$ ) and indicates that the actual number of viable phage containing insert DNA is  $6 \times 10^5$ .

Additional proof that X. campestris DNA had been successfully cloned was obtained by isolating the DNA present in six independent clones that grew on KM392 and Q359 but not on KRO. The size of each isolated phage chromosome was found to be slightly greater than the size of the lambda 1059 chromosome, indicating that the insert present in each clone is larger than the 14 kb "stuffer" fragment. A BamHI digest of the DNA from each of the isolates showed that each had a unique restriction pattern that was different from the BamHI restriction pattern of lambda 1059.

#### Example 4

This example describes the screening of the library of X. campestris DNA and demonstrates that some of the genes involved in xanthan biosynthesis are clustered.

Mutants of X. campestris that do not produce xanthan gum were isolated using Tn10 mutagenesis. The tetracycline resistance encoded by Tn10 has been used to clone restriction endonuclease fragments containing Tn10 and the chromosomal DNA from 35 gum::Tn10 mutants. These chromosomal sequences that flank the Tn10 insertion site provided hybridization probes used to identify wild-type X. campestris DNA sequences cloned in the lambda genomic library and to produce a physical map of gum::Tn10 mutations.

Chromosomal DNA was extracted and purified from the gum::Tn10 mutants as described above in Example 3. This DNA was digested to completion with restriction endonuclease PstI. This enzyme does not cleave within Tn10. Therefore, one chromosomal PstI fragment will contain the Tn10 element intact and fused to the X. campestris chromosomal DNA adjacent to the insertion site. The digested DNA was ligated to PstI-digested plasmid RSF1010 as follows.

Digestions were monitored by running aliquots of the reactions on agarose gels. In a typical ligation reaction, approximately 8 ug of plasmid and approximately 5 ug of chromosomal DNA were combined in a total volume of 200 ul. Approximately 20 units of T4 DNA ligase (New England Biolabs) were added and reactions were incubated at 12°C for approximately 16 hours. The extent of ligation was assayed by agarose gel electrophoresis of an aliquot of the reaction prior to transformation.

Ligation products were used to transform *E. coli* LE392, selecting for resistance to streptomycin (carried by RSF1010) or tetracycline. The entire ligation reaction was then used to transform *E. coli* LE392. Selection for Tet<sup>r</sup> transformants should select for recombinant plasmids that contain both the large *Pst*I fragment of RSF1010 (which provides replication function) and the *Pst*I fragment of chromosomal DNA that contains Tn10 (which provides tetracycline resistance). For the transformation procedure, the ligation reaction is ethanol precipitated, resuspended in 50  $\mu$ l of buffer, and added to 0.2 to 1.0 ml of cells (made transformation-competent by CaCl<sub>2</sub> treatment) at a concentration of about  $3 \times 10^9$  per ml. This mixture is incubated on ice for 45 minutes, heat-shocked at 43°C for two minutes, diluted five-fold with Luria broth, incubated at 37°C for 60 minutes, and finally concentrated and plated on the appropriate drug-containing medium.

All ligations with chromosomal DNA's containing Tn10 gave some tetracycline-resistant transformants which contained recombinant plasmids carrying cloned Tn10 DNA. No tetracycline-resistant transformants were obtained from control transformations with RSF1010 alone, with no DNA, and, most significantly, with a ligation reaction using chromosomal DNA extracted from S4-L str-101 which does not carry Tn10. The frequency of streptomycin-resistant transformants in this control ligation was, however, equivalent to the frequencies of streptomycin-resistant transformants obtained from the other ligations.

Tetracycline-resistant transformants were analyzed for the presence of recombinant plasmids carrying Tn10. Colonies were picked and grown overnight in LB with 10  $\mu$ g/ml tetracycline. Plasmid DNA was prepared using a standard, cleared lysate technique as described by Clewell and Hsiung in *Biochemistry* 62:1159-1166 (1969), specifically incorporated herein by reference. Plasmids were analyzed by digestion with appropriate restriction endonucleases. In order to test for the presence of Tn10 on the plasmid, a *Hind*III digest was done. Tn10 contains two internal *Hind*III fragments, 4.8 kb and 0.5 kb in length. Thus, all recombinant plasmids should generate these two fragments when cut with *Hind*III. Additionally, when cut with *Pst*I, all recombinants should yield an 8.1 kb fragment derived from RSF1010, plus a second, larger fragment which ought to be greater than 9.3 kb. This is the *Pst*I fragment of chromosomal DNA that contains Tn10, which is itself 9.3 kb in length.

Nearly all plasmids examined appeared to contain Tn10 by these criteria. However, *Pst*I digests often revealed the presence of more than two *Pst*I fragments. Presumably, these "extra" fragments are the result of multiple ligation events. These extra fragments are undesirable because, if such a recombinant were used as a hybridization probe, annealing to DNA fragments homologous to the extraneous *Pst*I fragment could occur, as well as annealing to the *Pst*I fragment that contains sequences from the gene of interest.

In order to eliminate the extraneous *Pst*I fragments, the Tn10-containing fragment was then "recloned." Purified plasmid DNA was prepared over CsCl gradients and digested to completion with *Pst*I. Digestion products were ligated at low DNA concentration (approximately 10  $\mu$ g/ml). At this relatively low concentration, most ligation events should circularize linear fragments. Occasional dimer circles will be formed and higher order multimers should be rare.

The extent of ligation was assayed by gel electrophoresis of an aliquot of the reaction, and, in general, little multimer formation could be observed. The ligation reaction was then used to transform *E. coli* LE392, and tetracycline-resistant transformants were selected. The plasmids present in these transformants were then analyzed; they were nearly always found to have the desired recombinant structure. That is, they contained two, and only two, *Pst*I fragments: one 8.1 kb in size, which is derived from RSF1010, and a second fragment greater than 9.3 kb in size, which is the *X. campestris* chromosomal fragment containing the Tn10 and the adjacent genomic DNA. These resulting recombinant plasmids were designated pTXnnn, where T stands for tetracycline resistance, and Xnnn is the strain number of the gum::Tn10 mutant from which the tetracycline resistance is cloned. For example, plasmid pTX655 was derived by cloning the tetracycline-resistance determinant out of gum::Tn10 mutant strain X655.

The Gum<sup>-</sup> mutant designated X655 produces a polysaccharide having subunits with a trimer structure instead of the normal pentamer structure. This mutant is described more fully by Vanderslice et al., *supra*. It seemed possible that the gum biosynthetic gene defined by this mutation might be part of a cluster of genes that are coordinately expressed and regulated to bring about gum biosynthesis. To test this possibility, a set of 26 lambda 1059 clones carrying *X. campestris* DNA that hybridizes with plasmid pTX655 (called henceforth lambda 655(+)) was isolated and purified. Since the gene bank had not been amplified, each clone contained *X. campestris* DNA derived from an independent ligation event. The effect of this was to "walk" along the chromosome in the region of the genome carrying the gum gene defined by the X655 mutation. Since each cloned fragment is approximately 15 kb, the "walk" covered about 30 kb.

The set of 26 phage clones was then hybridized in turn with probe DNA cloned from each of 35 gum::Tn10 mutants. Twenty-four of thirty-five plasmid probes were found to hybridize with either all or some of the 26 phage clones in the set. These results indicate that at least some of the gum genes are

clustered in the same region of the *X. campestris* genome that contains the gum biosynthetic gene defined by the X655 mutation, Transferase IV.

The 26 lambda 655(+) recombinant phages contain, as a population, approximately 30 kb of chromosomal *X. campestris* DNA centered around the *Pst*I fragment cloned in pTX655. By hybridizing the other 34 pTX plasmids against these phages, it was determined which of the other Gum<sup>-</sup> mutants had Tn10 insertions within this 30 kb segment. All 35 pTX plasmids were radiolabeled by nick-translation and hybridized to filter-bound lambda 655(+) phage DNA along with lambda 1059 cloning vector DNA, which served as a negative control.

Nick translations were carried out in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 mM DTT, and 50 ug/ml BSA (Sigma Pentax Fraction V). Typical reaction volume was 30 ul and generally 0.4 ug of DNA was added. Cold dNTP's were each present at 20 uM, and <sup>32</sup>P-labeled dNTP's were each present at 3.3 uM. A total of approximately 80 uCi of <sup>32</sup>P was added with each hot dNTP; generally one or two nucleotides were labeled. This reaction mixture was treated with DNAase I (1 uliter of a 0.1 ug/ml solution) for 1 minute at 37°C. Subsequently, 1 ul of *E. coli* DNA polymerase I (5 units as defined by Richardson et al. (1964)) was added to the reaction. After incubation at 37°C for 30 minutes, the reaction was ethanol precipitated with carrier DNA. The pellet was washed with 70% ethanol, vacuum-dried, and resuspended in 200 ul 10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA.

Hybridizations were conducted by the following method. Nitrocellulose filter bound lambda DNA's were prepared using the protocols of Davis et al. (1980). These filters were prehybridized in 5x SSPE, 5x Denhardt's solution (Maniatis et al. supra), 0.1% SDS, and 50% formamide containing 100 ug/ml denatured, sonicated calf thymus DNA for 4-16 hours at 42°C on a rocker. The hybridization reaction itself was done in 2x SSPE, 1x Denhardt's solution, 50% formamide containing 100 ug/ml denatured, sonicated calf thymus DNA. Radiolabeled <sup>32</sup>p probe DNA's were denatured in 0.1 M NaOH and neutralized by addition of 1/10 volume of 2M Tris-HCl (pH 8.0). Typically, 10<sup>6</sup> cpm of incorporated <sup>32</sup>P were added per ml of hybridization reaction. Hybridizations were incubated at 42°C for 12-20 hours on a rocker. Subsequently, filters were washed at room temperature, once in 2x SSPE and then once in 0.1x SSPE. Filters were blotted on Whatman 3 MM paper and allowed to air dry. The filters were then placed under Kodak X-OMAT AR film and exposed for 4-16 hours at -70°C. A Du Pont Coronex intensifying screen was employed.

It was found that 25 of the 35 plasmids hybridized to some or all of the lambda 655(+) phage DNA's. Ten probes failed to hybridize to any of the lambda 655(+) phage. Thus, a sizable fraction of the gum::Tn10 mutations (approximately 60%) are located within this cloned 30 kb region, but a significant number lie outside this DNA segment.

The hybridization data allow classification of the lambda 655(+) phage on the basis of which probes hybridized and which failed to hybridize. These hybridization patterns reflect the DNA segments cloned in each phage. Because each of the cloned DNA fragments is a single contiguous piece of the *X. campestris* chromosome, the order of the mutations in the genome was deduced from the classes of hybridization patterns. The presence or absence of particular DNA fragments correlates well with hybridization to, or failure to hybridize to, particular probes.

The restriction maps and hybridization data indicated that the mutant X708 was located quite near one end of the cloned 30 kb segment of *X. campestris* DNA. The *X. campestris* chromosome was thus "walked" along by isolating recombinant lambda phage that hybridized to the pTX708 plasmid. Twenty-six such recombinants were picked and analyzed by restriction mapping as described above. This set of phage extended the cloned region by approximately 8 kb.

All 35 pTX plasmid probes were then hybridized to this set of recombinant phage using the same procedures employed in hybridizations with the lambda 655(+) phage. The same 25 pTX probes annealed to some or all of the lambda 708(+) phage, and the same set of probes that failed to hybridize to any lambda 655(+) phage also failed to hybridize to any of the lambda 708(+) phage. Again, the restriction maps of the lambda 708(+) phages permitted correlation of the presence or absence of particular DNA fragments with hybridization to particular plasmid probes. In summary, then, from these hybridizations, 25 mutations were found to be clustered in a region of DNA in the near vicinity of the mutation carried in the Transferase IV- strain X655. Ten mutations did not map to this region of DNA. Some of these mutations are described above and by Betlach et al., supra. These mutations do not alter the gum biosynthetic enzymes.

#### Example 5

This example describes the restriction mapping of cloned DNA carrying clustered gum biosynthetic genes.

Since a large number of the gum genes defined by the collection of Tn10 insertional mutations were found to be clustered around the location of X655 mutation, the region of the *X. campestris* genome carrying these genes was further characterized by restriction enzyme mapping. This was accomplished by generating a restriction enzyme map of the cloned DNA present in each of the 26 phage clones constituting the set of overlapping fragments carrying the gum gene cluster.

Since each of the phage clones contained lambda 1059 DNA in addition to the cloned *X. campestris* DNA, the restriction enzyme analysis was done with a restriction enzyme that would readily permit the distinction of the lambda DNA from the *X. campestris* DNA. One such enzyme is *Bam*HI. Because there are no *Bam*HI sites in either of the two arms of lambda 1059, the lambda DNA present in each *Bam*HI digest will always be located in two bands: one greater than or equal to 20 kb contains lambda DNA from the left arm, the other greater than or equal to 9 kb contains lambda DNA from the right arm.

DNA isolated from each of the 26 phage clones was digested with *Bam*HI. Since the restriction fragments produced ranged in size from greater than 20 kb to less than 0.5 kb, the digests were run on low percentage agarose gels (to separate the large fragments) and at high voltage (to reduce diffusion of the smallest fragments). The gels often used an agarose concentration of 0.4% run at 100 volts for about 5 hours. Each gel contained samples of the phage clones digested with *Bam*HI, a *Hind*III digest of wild-type lambda DNA (for use as a size standard), and a *Bam*HI digest of lambda 1059 DNA (to mark the location of vector DNA in the sample digests). The distance migrated by each restriction fragment in the sample digests was measured and converted to a molecular size using a standard curve prepared from the *Hind*III digest of wild-type lambda DNA.

The restriction patterns generated by the *Bam*HI digests of the DNA present in each of the 26 phage clones were analyzed to determine the regions of overlapping DNA in each of the phage clones. From the pattern of overlaps, the order of the restriction fragments in each of the digests was determined (Figure 2).

Since the position of the X708 mutation was very close to one end of the cloned DNA, it seemed possible that one or more of the 10 pTX probe DNA's that did not hybridize was located just outside of the region of the DNA contained in the set of overlapping phage clones. This possibility was tested by isolating a set of lambda clones which hybridize with pTX708 from the gene bank. In this way, the region of the *X. campestris* genome contiguous with the gum gene cluster was extended beyond the location of the Gum<sup>-</sup> mutation present in X708. *Bam*HI restriction maps were prepared for the DNA contained in these clones (Figure 3).

To determine if there were any very small *Bam*HI restriction fragments (less than 0.5 kb) that were not detected on 0.4% agarose gels, selected phage clones containing DNA derived from the entire cloned region were digested with *Bam*HI and run on a 5% polyacrylamide gel. Such a gel can resolve fragments as small as 30 bp. This experiment revealed the presence of two previously undetected *Bam*HI fragments having sizes of 300 bp and 190 bp.

Further analysis of these data indicated that the 300 bp fragment is located between the 1.05 fragment and 1.4 kb fragment, while the 190 bp fragment is located between the 5.8 kb fragment and 1.05 kb fragment. All of the *Bam*HI restriction mapping data and the probe hybridization data were combined to generate a physical and genetic map of a region of the *X. campestris* chromosome that carries a cluster of genes involved in xanthan gum biosynthesis (Figure 4).

#### Example 6

Example 6 describes the subcloning of gum gene cluster DNA from the lambda 1059 library onto the broad-host range plasmid vector pMW79.

The vector pMW79 is described in detail by Wood *et al.* in J. Bact. 14:1448-1451 (1981), specifically incorporated herein by reference. Briefly, it is a chimeric plasmid combining the broad-host range, Inc-Q plasmid RSF1010 and the classical *E. coli* cloning vector pBR322 (see Figure 5a). pMW79 can be transferred to, and propagated in, a wide variety of gram-negative bacteria and retains many of the useful cloning sites present in pBR322. Cloning sites in the pBR322 portion of the plasmid have been principally used and in particular sites have been used that occur within the tetracycline resistance gene.

The lambda 1059 phage clone 655 (I) contains the entire region of the *X. campestris* genome that carries the gum genes defined by our Tn10 mutations. *X. campestris* DNA in phage clone I was subcloned into the plasmid cloning vector pMW79.

A partial *Bam*HI digest of DNA isolated from lambda clone 655 (I) was ligated with a *Bam*HI limit digest of pBR322 and pMW79. The ligated DNA was used to transform KM392 selecting Amp<sup>r</sup> transformants. A total of 1200 Amp<sup>r</sup> transformants were printed on agar plates containing tetracycline to determine which of the transformants are Tet<sup>s</sup> and, thus, likely to contain an insert in the unique *Bam*HI site located in the Tet

gene of pMW79. Sixty Amp<sup>r</sup> Tet<sup>s</sup> transformants were isolated and streak purified. Plasmid DNA was then isolated from each of the transformants, digested with BamHI, and the digests were run on 0.4% agarose gels to ascertain the presence and extent of X. campestris DNA.

The majority of the isolated plasmids contained no insert DNA. However, 19 were found that did contain  
 5 inserts. Nine of the 19 contained a single fragment insert, while 10 contained inserts composed of two or more fragments. In all 10 cases where two or more BamHI fragments are present in the cloned DNA, the fragments are contiguous on the BamHI restriction map of lambda clone 655 (I). This finding provides independent evidence that the order of the BamHI fragments in lambda clone 655 (I) is correct. With the exception of the 1.0 kb fragment on the right end, all of the Xanthomonas DNA present in lambda clone  
 10 655 (I) is represented in one or more of the subclone derivatives.

Clones of pMW79 containing either the 5.8 kb fragment or the 11.5 kb fragment (which are not contained in lambda clone 655 (I) were prepared in a separate experiment by ligating BamHI limit digests of lambda clone 655 (C') and lambda clone 708 (8) with BamHI-digested pMW79. A representative sample of X. campestris DNA fragments that were thus cloned into pMW79 is shown in Figure 5b.

In a series of steps, a single large (20 kb) segment of DNA spanning the region that is genetically implicated in xanthan gum biosynthesis was cloned into pMW79. Steps followed in this cloning were as follows. Phage lambda clone 655(B) (Figure 2) DNA was digested to completion with BglII and HindIII and ligated with plasmid pMW79 DNA digested to completion with BamHI and HindIII. BamHI and BglII cut different sequences but create identical cohesive ends; thus, BamHI ends can be ligated to BglII ends. The  
 15 ligation products were used to transform E. coli selecting for ampicillin resistance encoded by pMW79. Eighty-eight Amp<sup>r</sup> transformants were screened for resistance to tetracycline and 75 Tet<sup>s</sup> isolates were found. Cloning into the BamHI-HindIII region of pMW79 results in inactivation of tetracycline resistance. The high frequency of Tet<sup>s</sup> transformants occurs because HindIII and BamHI ends cannot be ligated together to reseal the plasmid; insertion of a DNA fragment is required for recircularization. Six Tet<sup>s</sup> transformants were  
 20 analyzed for plasmid. Small-scale cleared lysates were prepared, digested with BamHI, and run out on agarose gels. Two transformants proved to have plasmids carrying the desired fragment. One isolate was grown up for a larger plasmid preparation. This plasmid DNA was purified by CsCl density gradient centrifugation and reanalyzed by various restriction endonuclease digestions. This plasmid (now termed pX206) had the expected structure, shown in Figure 6, as evidenced by the cutting patterns of these  
 25 restriction endonucleases.

An attempt was also made to clone the 8 kb BglII fragment of lambda 708(8) as shown in Figure 6. Purified lambda 708(8) DNA was digested to completion with BglII and ligated with BamHI-cut pMW79 DNA. The ligation mixture was used to transform E. coli, again selecting for Amp<sup>r</sup>. One hundred sixty-six  
 35 Amp<sup>r</sup> transformants were tested for resistance to tetracycline and 49 Tet<sup>s</sup> isolates were found. Plasmid DNA from 18 of these was isolated and one recombinant plasmid that appeared to carry the BglII fragment of interest was found. A large-scale cleared lysate was prepared from this strain and purified the plasmid DNA over CsCl gradients. Further analysis indicated that this plasmid had the fragment of interest but contained a second, extraneous BglII fragment as well.

The structure of this fortuitous recombinant provided an opportunity to construct a more useful  
 40 subclone. By chance, the locations of the BglII and ClaI sites in this plasmid provided an opportunity to digest with ClaI and BglII and then ligate in the 2 kb ClaI-BglII fragment that is contiguous in the gum gene cluster. This construction extends the cloned DNA present in the plasmid by 2 kb beyond the BglII site; this extra DNA was useful in facilitating gene replacement experiments. Additionally, this construct contained unique HindIII and BglII sites within the cloned Xanthomonas DNA. The structure of this plasmid, designated  
 45 pX207, is shown in Figure 6.

Subsequently, the large (8 kb) BglII-ClaI fragment of lambda 655(B) was inserted into pX207, replacing the small (2 kb) BglII-ClaI segment of pX207. Plasmid pX207 was digested to completion with both these enzymes and ligated with the DNA of the lambda recombinant lambda 655(B), which was also digested with  
 50 BglII and ClaI. The double digestion with ClaI and BglII selects for recombinant plasmids among the transformants because BglII ends cannot be ligated to ClaI ends, and thus the pX207 plasmid cannot recircularize unless a second BglII-ClaI fragment is ligated into it. Indeed, the 12 transformants that were examined all contained recombinant plasmids, and one of these proved to be the desired recombinant. This plasmid, pX208, is shown in Figure 6. This plasmid contains the gum gene DNA from the right-hand BglII site of the 5.8 kb BamII fragment through the ClaI site of 11.5 kb BamHI fragment, with the exception of an  
 55 interstitial 4.5 kb BglII piece. The missing 4.5 kb BglII fragment was subsequently inserted into pX208 to create the large subclone of interest, termed pX209.

The pX208 plasmid DNA was linearized by digestion with BglII. The missing 4.5 kb BglII fragment was purified by electroelution out of a preparative agarose gel and ligated to the BglII-cut pX208. Ligation

products were used to transform *E. coli* and ampicillin-resistant transformants were obtained. In this ligation, there is no selection for recombinants and there is no simple screening procedure. The transformants were screened for recombinant plasmids by the technique of colony hybridization (Maniatis *et al.*, *supra*). This procedure is analogous to the plaque hybridization protocol used to screen lambda clones for DNA segments of interest. Transformants are toothpicked onto a "master" plate in an ordered array. This master plate is then used to produce a copy on a nitrocellulose filter. This filter copy is incubated on top of an agar plate with the result that bacterial growth occurs on the surface of the filter, fed by diffusion of nutrients from the agar through the filter. Subsequently, the bacteria on the filter are lysed *in situ* and DNA is irreversibly bound to the filter. This filter can then be probed with any radiolabeled DNA. In this instance, a radiolabeled 4.5 kb *Bgl*III fragment was used; only recombinants that acquired this fragment hybridized to the probe. Most transformants contained only the recircularized plasmid pX208 and did not hybridize. Five hundred and seventy-six transformants were screened using the 4.5 kb *Bgl*III DNA labeled with <sup>32</sup>P by the nick translation procedure. Among these, 20 transformants were found that hybridized to the probe. Plasmid DNA's from some of these transformants were analyzed in order to verify the presence of the 4.5 kb fragment and determine its orientation. Plasmids from twelve such putative recombinants were analyzed using agarose gel electrophoresis. Eleven of these contained the expected *Bgl*III 4.5 kb fragment, and, of these eleven, eight carried the fragment in the correct orientation. One of these eight was picked for further analysis. This plasmid, designated pX209, is depicted in Figure 6.

## 20 Example 7

This example describes methodology for *in vivo* and *in vitro* regionally-directed mutagenesis of the cloned gum gene DNA segment carried on pMW79.

Regionally-directed mutagenesis was performed upon subcloned portions of the gum DNA carried in plasmid pMW79. These cloned DNA segments were mutagenized *in vivo* with transposons and *in vitro*, by using recombinant DNA technology to generate insertion, deletion, and substitution mutations within the cloned *X. campestris* DNA. In order to study the phenotypes conferred by these mutations, the plasmids carrying the mutations were transferred back into *X. campestris* and subsequently recombinants were identified in which the plasmid-borne, mutated gene had been inserted in the chromosome via homologous recombination. The tetracycline resistance encoded by Tn10 affords a convenient selective system for movement of mutations from a plasmid into the chromosome.

In preliminary experiments designed to study recombination between plasmid-borne *X. campestris* DNA and the *X. campestris* chromosome, the plasmid pTX655 was used as a model system. This plasmid carries a Tn10 insert in the middle of a 2.3 kb *X. campestris* PstI fragment cloned in plasmid RSF1010. The experiment was to mobilize pTX655 with plasmid pRK2013 and transfer it from *E. coli* into *X. campestris* by selecting for movement of the tetracycline resistance encoded by Tn10. The initial results of this mating were anomalous and suggested that Tn10 did not express tetracycline resistance efficiently in *X. campestris* when carried on the plasmid, but that the drug resistance was more efficiently expressed when Tn10 was carried in the chromosome of *X. campestris*. This phenomenon has also been described for Tn10 in *E. coli*. There, it has been shown that strains carrying one copy of Tn10 inserted in the chromosome are resistant to significantly higher concentrations of tetracycline than are strains carrying Tn10 on a multicopy plasmid. The selection of Tet<sup>r</sup> *X. campestris* out of the above mating resulted in a high frequency (0.5 per recipient) of progeny which grew very poorly (i.e., only small, watery colonies) on tetracycline. After prolonged incubation, a large fraction of the colonies (25%) produced sectors of more vigorously growing cells. More than 50% of these sectors appeared to be Gum<sup>-</sup> in morphology. These probably result from recombination between the plasmid-borne DNA containing the Tn10 insertion and the chromosomal wild type DNA. When the Tn10 is recombined into the chromosome, high-level Tet<sup>r</sup> is obtained and the vigorously growing sector is observed. When these Gum<sup>-</sup> Tet<sup>r</sup> sectors were picked and restreaked on tetracycline, they grew well and displayed a characteristic Gum<sup>-</sup> morphology. This strongly argues that the original X655 mutation has been reconstituted by recombination of the plasmid-carried Tn10 insertion into the chromosome.

Lambda 173, as described by Kleckner *et al.* in Genetics 90:426-461 (1978), specifically incorporated herein by reference, was used to introduce Tn10 into plasmids containing *X. campestris* DNA. This bacteriophage contains a temperature-sensitive repressor of lytic functions and a Tn10 insertion in a nonessential gene. Aliquots of this phage were used to infect a lambda-sensitive *E. coli* carrying a recombinant plasmid of interest at multiplicity of infection of 0.1 at 30°C. After 45 minutes (to allow phage adsorption, DNA injection, and expression of tetracycline resistance), the cells are pelleted to remove any unadsorbed phage. Then the cells are resuspended in Luria broth and aliquots containing 10<sup>8</sup> cells are plated on Luria plates containing 20 ug/ml tetracycline, 100 ug/ml ampicillin, and 2.5 mM sodium

pyrophosphate. The tetracycline selects for Tn10, while the pyrophosphate chelates magnesium to minimize secondary phage infections. The formation of lysogens is minimized by incubating the plates at 42°C. Tet<sup>r</sup> survivors arise at a frequency of 10<sup>-7</sup> to 10<sup>-6</sup>. In an experiment designed for the purpose of plasmid mutagenesis, 10<sup>10</sup> cells are infected with 10<sup>9</sup> phage, and 10<sup>2</sup> plates are spread. After 24-hour incubation, the colonies from each plate are suspended in 4 ml Luria broth and pooled on ice. After centrifugation, plasmids are extracted from the cells using a lysozyme-Triton cleared lysate protocol, and then plasmid DNA is purified by ethidium bromide-caesium chloride density gradient centrifugation. After further purification, the plasmid DNA is used to transform *E. coli* LE392 with selection for Amp<sup>r</sup>, Tet<sup>r</sup>. Subsequently, plasmid DNA is extracted from each transformant and cut with *Bam*HI to localize the insertion to *X. campestris* DNA or vector DNA. Those plasmids with Tn10 inserted in *X. campestris* sequences are then mobilized into Gum + *X. campestris*. Selection for tetracycline-resistant *X. campestris* out of this mating frequently results in movement of the plasmid-borne Tn10 insertion into the chromosome of *X. campestris* via homologous recombination, as detailed above. The phenotypic properties of *X. campestris* strains carrying these chromosomal insertions can then be analyzed.

A second strategy for isolating Tn10 insertion *in vivo* was also employed. This strategy used plasmid pRK2013::Tn10 described in Example 1 as a source of Tn10. Advantage was taken of the incompatibility between the Amp<sup>r</sup> pMW79 replicon (of pX113) and pRK2013::Tn10, and the lack of a *Sma*I restriction site in pX113. Rif<sup>r</sup> Amp<sup>r</sup> *E. coli* (pX113) was mated with Rif<sup>r</sup> *E. coli* (pRK2013::Tn10), with selection for Rif<sup>r</sup> Amp<sup>r</sup> Tet<sup>r</sup>. The selection for Amp<sup>r</sup> Tet<sup>r</sup> with the plasmid incompatibility should favor cells which sustain transpositions of Tn10 onto pX113. Plasmid DNA was purified from a pool of several thousand colonies and then cut to completion with restriction endonuclease *Sma*I. Plasmid pRK2013::Tn10 has *Sma*I sites within the vector portion of the plasmid, while there are no *Sma*I sites within Tn10. The resultant DNA was used to transform a native *E. coli* to Amp<sup>r</sup> Tet<sup>r</sup>. Only pX113::Tn10 plasmids should confer this phenotype during transformation. The plasmid content of Amp<sup>r</sup> Tet<sup>r</sup> transformants was analyzed by *Bam*HI restriction endonuclease cutting and agarose gel electrophoresis. Some of them contained inserts of Tn10 within the cloned *Xanthomonas campestris* sequences. Such plasmid-borne insertion mutations could be introduced into the *X. campestris* chromosome via gene replacement as described above.

The *in vitro* mutagenesis experiments were also designed to exploit the useful properties of the Tn10 tetracycline resistance determinant. A 2.8 kb *Bgl*II fragment was purified from Tn10. Previous work has shown that this fragment contains intact the gene encoding the protein conferring tetracycline resistance and a regulatory gene which encodes a protein that regulates the expression of the resistance gene. In *E. coli*, at least, this regulatory gene must be functional for the difference between plasmid-borne and chromosomally-located tetracycline resistance to be observed. The DNA fragment of interest was purified from preparative agarose gels by electrophoretic elution of DNA out of gel slices (Maniatis *et al.*, 1982). The eluted DNA was extracted with phenol two times, ethanol-precipitated, washed with 70% ethanol, vacuum dried, and resuspended in appropriate buffer.

*In vitro* insertions of this DNA fragment were subsequently made in *Bgl*II and *Bam*HI sites present within cloned gum gene DNA carried on various pMW79 derivatives. In making these new constructs, advantage was taken of the fact that the cohesive ends of *Bam*HI-cut DNA are identical to the cohesive ends of *Bgl*II-digested DNA; therefore, *Bgl*II-cut DNA can be ligated into a *Bam*HI site. Plasmid DNAs were digested with *Bam*HI in the presence of 20-80 µg/ml of ethidium bromide. In the presence of ethidium bromide, the activity of the restriction endonuclease is perturbed. The result is that this digestion produces a high proportion of singly-cut linear products. A priori, one would expect that the *Bam*HI site that is cut would be chosen, more or less, at random and, to a first approximation, this appears to be so. The purified *Bgl*II Tet<sup>r</sup> fragment can be ligated to this population of linear fragments. The ligation products were used to transform *E. coli* and tetracycline-resistant transformants were selected. Plasmids were extracted from these transformants and analyzed by restriction endonuclease digestion. Plasmids carrying an insertion of the *Bgl*II Tet<sup>r</sup> fragment at either a *Bam*HI or *Bgl*II site were then mated into *X. campestris* and the differential tetracycline resistance of the chromosomal vs. plasmid-borne Tet<sup>r</sup> element was used to identify homologous recombination events that generate gene replacements. With slight modification of this technology, deletion mutations were also constructed. When the plasmid DNA's digested in the presence of ethidium bromide incurred two or more cleavages by *Bam*HI, the segment flanked by the two cleaved sites was lost. When the Tet<sup>r</sup> *Bgl*II fragment was ligated to such linear plasmid molecules, the resultant recombinant contained a deletion of a segment of gum gene DNA. Plasmids containing deletions of gum gene DNA were also purposely constructed by ligating together noncontiguous segments of gum gene DNA. If the Tet<sup>r</sup> *Bgl*II fragment is present at the junction to two such cloned, noncontiguous gum gene cluster DNA segments, it often proved possible to introduce even very large deletions into the *Xanthomonas campestris* chromosome via gene replacement. For example, the deletion strain X1107, which deletes 15 kb of DNA from the gum

gene cluster, was constructed by this technique.

Through the application of both in vivo and in vitro regionally-directed mutagenesis technology to a number of subcloned segments of gum gene DNA, numerous plasmid-borne insertions were obtained which subsequently gave rise to tetracycline-resistant X. campestris derivatives in the gene replacement experiment. A physical map of some of these insertion mutations is shown in Figure 7. Southern blot hybridization analyses of the chromosomal DNA's of these gene replacement strains was performed in order to confirm that the positions of the chromosomal insertions corresponded to the positions of the plasmid-borne insertions from which they were derived. For these experiments, total chromosomal DNA was prepared from each putative gene replacement strain as described above. The purified DNA's were digested with diagnostic restriction endonucleases; usually BamHI, BglII, EcoRI, HindIII, or PstI, or some combination of the above. DNA digests were run out on agarose gels, transferred to nitrocellulose filters, and probed with radiolabeled plasmid DNA. Autoradiographs reveal the pattern of hybridization, which is compared to wild-type controls to deduce the location and orientation of the Tn10, or BglII Tet<sup>r</sup> fragment, insertion. All of the insertions shown in Figure 7 were found to be genuine gene replacement strains. That is, no plasmid sequences were detected by hybridization and the chromosomal DNA's were altered from the wild-type by the indicated insertion mutation and by no other evident event. The phenotypes of all these insertion mutations were examined by in vitro and/or in vivo methodologies described in Example 2. The phenotypes thus identified are indicated on Figure 7.

#### 20 Example 8

This example describes procedures used in complementation experiments that demonstrate the expression, in X. campestris, of cloned gum biosynthetic genes carried on plasmid vectors.

Complementation experiments have been performed with plasmids carrying segments of gum gene DNA and Gum<sup>-</sup> insertion mutations within the gum gene cluster. The results of these experiments are summarized in Figure 8. In these experiments, the plasmids were mated into X. campestris recipients and maintained there using selection for streptomycin resistance. The presence of plasmid was confirmed by making plasmid DNA preps out of each recipient strain and analyzing the plasmid DNA with restriction endonuclease digestion and agarose gel electrophoresis. Plasmid "curing" experiments were performed to demonstrate that loss of the plasmid was correlated to loss of the Gum<sup>+</sup> phenotype in complemented strains. In general, mutants were complemented by plasmid DNA's that spanned the mutational insertion site. For example, plasmid pX110 complemented X925, X928, X975, and X855 but not the deletion mutant strain X974. The gum gene DNA carried by plasmid pX110 extends significantly beyond the insertion sites of all the complemented mutations. However, as seen in Figure 8, the deletion mutation in X974 extends right up to the right-hand endpoint of cloned gum DNA carried in pX110. It is possible, therefore, that pX110 does not carry an intact copy of the gene eliminated by the deletion in strain X974. In fact, the failure to obtain complementation of X974 by pX110 argues that this is the case.

A surprising result was the failure of pX206 to complement X974. This plasmid also fails to complement X975, but the cloned segment extends only 1.0 kb beyond the site of the X975 insertion. It is entirely plausible that some element (e.g., a promoter) that is required for expression of the gene that is mutated in X975 is located outside the cloned segment in pX206. The failure of pX206 to complement X974 is more perplexing since the cloned gum gene DNA extends 4 to 5 kb beyond the mutation site in both directions. However, this particular mating was unique in that it gave an unusually low frequency of plasmid transfer. This frequency was three orders of magnitude lower than that observed in all other matings in this set of experiments. In all other matings, the plasmid pX206 was transferred at the higher frequency, and in all other matings with X974 as the recipient, the higher transfer frequency was observed. Thus, there may be a negative impact resulting from combination of that particular chromosomal mutation and that particular cloned DNA segment.

Most of the complementation results can be interpreted in a straightforward manner. The plasmid-borne gum genes can be expressed at least in X. campestris.

#### Example 9

This example illustrates the sequencing of DNA from the gum gene biosynthetic cluster.

The nucleotide sequence of the cloned segment of DNA containing the gum gene cluster is being determined using the dideoxy chain termination sequencing procedures described by Sanger et al. in Proc. Natl. Acad. Sci. USA 74:5463-5468 (1977) specifically incorporated herein by reference. Each of the BamHI fragments contained in the gum gene cluster have been cloned (in both orientations) into the M13 cloning

vectors MP18 or MP19. Each cloned fragment was then subcloned using a procedure described by S. Henikoff in Gene 28:351-359 (1984) specifically incorporated herein by reference. By sequencing the DNA contained in a nested set of overlapping subclones, a nucleotide sequence for the entire cloned BamHI fragment can be obtained. Thus far, a complete sequence of each of the two DNA strands has been

5 determined for the 0.3, 1.4, 1.5, and 2.2 kb BamHI fragments as depicted below:

The 0.3, 1.4 and 1.5 kb fragments are as follows:

|    |            |                |            |             |            |            |
|----|------------|----------------|------------|-------------|------------|------------|
|    | 10         | 20             | 30         | 40          | 50         | 60         |
| 10 | GGATCCG    | GTT GAGGCGGTAA | CAGGGGATTG | GCATGGCATT  | GACGAAAGCG | GAGATGGCCG |
|    | 70         | 80             | 90         | 100         | 110        | 120        |
| 15 | AGCGTCTGTT | CGACGAAGTC     | GGCCTGAACA | AGCGTGAGGC  | GAAGGAATTC | GTCGACGCGT |
|    | 130        | 140            | 150        | 160         | 170        | 180        |
| 20 | TTTTCGATGT | GCTGCCGATG     | CACTGGAGCA | GGGCCGTGCA  | GGTGAAGTTG | TCGGGCTTGC |
|    | 190        | 200            | 210        | 220         | 230        | 240        |
|    | CGAACTTCGA | TCTGCGGCGC     | AAGAACCAAC | GGCCCCGGTCG | CAATCCCAAG | ACCGGTGAGG |
| 25 | 250        | 260            | 270        | 280         | 290        | 300        |
|    | AAATTCCGAT | CTTGGCCAGG     | ACGGTGGTGA | CCTTCCGCCC  | CGGCCAGAAA | CTCCAAGGAA |
|    | 310        | 320            | 330        | 340         | 350        | 360        |
| 30 | CGGGTGGAGG | CTTTATGCTG     | GATCCGGGCA | GTAATCGCGA  | GCTACCGCCG | ATTCCGGCCA |
|    | 370        | 380            | 390        | 400         | 410        | 420        |
| 35 | AGCGCTACTT | CACCATCGGT     | GAGGTGAGCG | AGCTGTGCGA  | CGTCAAGCCG | CACGTGCTGC |

40

45

50

55

|    |            |            |            |             |            |            |
|----|------------|------------|------------|-------------|------------|------------|
|    | 430        | 440        | 450        | 460         | 470        | 480        |
|    | GCTATTGGGA | AACCGAATTT | CCGAGCCTGG | AGGCCAGTCA  | AGCGGCGCGC | AACCGACGCT |
| 5  |            |            |            |             |            |            |
|    | 490        | 500        | 510        | 520         | 530        | 540        |
|    | ACTACCAGCG | GCACGATGTC | GTGATGGTGC | GGCAGATTTCG | TGGCCTGCTG | TACGAGCAGG |
| 10 |            |            |            |             |            |            |
|    | 550        | 560        | 570        | 580         | 590        | 600        |
|    | GTTACACCAT | CGGGGGCGCG | CGTCTGCGTC | TTGAAGGGGA  | TGGGGCCAAG | AGCGAGTCAG |
| 15 |            |            |            |             |            |            |
|    | 610        | 620        | 630        | 640         | 650        | 660        |
|    | CGCTGAGCAA | TCAGATCATC | AAGCAGGTGC | GCATGGAGCT  | TGAAGAAGTC | CTGCAGCTGC |
| 20 |            |            |            |             |            |            |
|    | 670        | 680        | 690        | 700         | 710        | 720        |
|    | TGCGACGCTA | GGAAAGCGCC | GCATAAAGCC | GCTATAATCG  | CAGGCCGCCT | CAGGGCGGGA |
| 25 |            |            |            |             |            |            |
|    | 730        | 740        | 750        | 760         | 770        | 780        |
|    | CGCAACATCT | TCGGGGTATA | GCGCAGCCTG | GTAGCGCACT  | AGTCTGGGGG | ACTAGTGGTC |
| 30 |            |            |            |             |            |            |
|    | 790        | 800        | 810        | 820         | 830        | 840        |
|    | GTCGGTTCGA | ATCCGGCTAC | CCCGACCAAA | CAACAGGCCT  | ACGTCGCAAG | ACGTGGGCCT |
| 35 |            |            |            |             |            |            |
|    | 850        | 860        | 870        | 880         | 890        | 900        |
|    | TTTTGTTGCG | TCGCAACATG | TCAGTTCGAT | GGCATTCCAG  | GCTATGCCAC | TATGCGCAAC |
| 40 |            |            |            |             |            |            |
|    | 910        | 920        | 930        | 940         | 950        | 960        |
|    | GGCATATTGC | AAGGCGGCAT | ATGCAAGTCC | TGTACGCAAT  | TATTTGCGCG | TTCAGGCTGC |
| 45 |            |            |            |             |            |            |
|    | 970        | 980        | 990        | 1000        | 1010       | 1020       |
|    | TACAAGTCGG | GATCAGCAGG | CGTCCGTAAG | TGCCCCGAAA  | CGCTAGAGTT | CGTATGCTGA |
| 50 |            |            |            |             |            |            |
|    | 1030       | 1040       | 1050       | 1060        | 1070       | 1080       |
|    | GAATGACGAC | CCAGGTCACG | TTCTCTTAAC | GTCGAGGCGA  | CGAACTTGAA | TCAATAGGCC |
| 55 |            |            |            |             |            |            |
|    | 1090       | 1100       | 1110       | 1120        | 1130       | 1140       |
|    | AACGCCGTCA | AAAAAATGGC | GTGTTGTGCC | TTGCGATGTG  | TTCGTTCTAT | GCCATAGTGC |
| 60 |            |            |            |             |            |            |
|    | 1150       | 1160       | 1170       | 1180        | 1190       | 1200       |
|    | ACTGCAACAC | GCGATTCAAC | GTTGGTCCCG | GCACGCGTCG  | GGATGCAACT | TCCTGTCGTA |

1210 1220 1230 1240 1250 1260  
 CGTTCGTGCT GGCGCCTGAG CCGGTTGAAT GCTGCGCGAG GTCCTGTCCC ACCCAACAGA  
 5  
 1270 1280 1290 1300 1310 1320  
 GGCAGCCAGC TACACGCATG AAGAAACTGA TCGGACGACT CGTCGCAAGG CCTCAGCCTG  
 10  
 1330 1340 1350 1360 1370 1380  
 GCTCTGCTCT GCTCGATGTC GCTGGGCGCT TGCAGCACCG GCCCGGAGAT GGCCTCTTCG  
 15  
 1390 1400 1410 1420 1430 1440  
 CTGCCGCATC CGGACCCGCT GGCAATGTCC ACGGTGCAGC CCGAATACCG TCTTGCGCCG  
 20  
 1450 1460 1470 1480 1490 1500  
 GGCGATCTGT TGCTGGTGAA GGTGTTTCAG ATCGACGATC TGGAGCGGCA GGTCCGCATC  
 25  
 1510 1520 1530 1540 1550 1560  
 GACCAGAACG GTCACATCTC ACTGCCGTTG ATTGGCGACG TCAAGGCCGC CCGTCTGGGC  
 30  
 1570 1580 1590 1600 1610 1620  
 GTTGGCGAAC TGGAAAAGCT GGTGCGCGAT CGGTATCGCG CAGGCTACCT GCAGCAGCCG  
 35  
 1630 1640 1650 1660 1670 1680  
 CAGATTTCCG TATTCGTGCA GGAGTCCAAC GGGCGTCGCG TCACGGTCAC TGGTGCGGTA  
 40  
 1690 1700 1710 1720 1730 1740  
 GACGAGCCGG GCATCTACCC GGTGATCGGC GCCAACCTCA CCTTGCAAGC GGCGATCGCG  
 45  
 1750 1760 1770 1780 1790 1800  
 CAGGCCAAGG GTGTCAGCAC GGTGGCAAGC CGCGGCAACG TGATCGTGTT CCGCATGGTC  
 50  
 1810 1820 1830 1840 1850 1860  
 AACGGGCAAA AAATGATTGC GCGGTTTCGAC CTGACCGAGA TCGAGAAGGG GGCCAATCCG  
 55  
 1870 1880 1890 1900 1910 1920  
 GATCCTGAGA TTTATGGCGG CGACATTGTC GTGGTGTATC GCTCGGATGC GCGCGTGTGG

|    |            |             |             |            |             |            |
|----|------------|-------------|-------------|------------|-------------|------------|
|    | 1930       | 1940        | 1950        | 1960       | 1970        | 1980       |
|    | TTGCGCACCA | TGCTGGAACT  | GACCCCCTTG  | GTGATGGTGT | GGCGCGCTTA  | CCGATGAGTA |
| 5  | 1990       | 2000        | 2010        | 2020       | 2030        | 2040       |
|    | TGAATTCAGA | CAATCGTTCC  | TCTTCGTCGC  | AGCGGTCATG | GTCATCTGGA  | ACTGGCAGAT |
| 10 | 2050       | 2060        | 2070        | 2080       | 2090        | 2100       |
|    | GTCGACTTGA | TGGACTACTG  | GCGCGCCCTG  | GTCTCGCAGC | TCTGGCTGAT  | CATCCTGATC |
| 15 | 2110       | 2120        | 2130        | 2140       | 2150        | 2160       |
|    | GCCGTCGGCG | CGCTGTTGCT  | GGCATTTCGGC | ATCACGATGT | TGATGCCCCGA | GAAGTACCGC |
| 20 | 2170       | 2180        | 2190        | 2200       | 2210        | 2220       |
|    | GCCACCAGCA | CCCTGCAGAT  | CGAACGTGAC  | TCGCTCAATG | TGGTGAACGT  | CGACAACCTG |
| 25 | 2230       | 2240        | 2250        | 2260       | 2270        | 2280       |
|    | ATGCCGGTGG | AATCGCCGCA  | GGATCGCGAT  | TTCTACCAGA | CCCAGTACCA  | GTTGCTGCAG |
| 30 | 2290       | 2300        | 2310        | 2320       | 2330        | 2340       |
|    | AGCCGTTTCG | TGGCGCGTGC  | GGTGATCCGG  | GAAGCCAAGC | TCGATCAGGA  | GCCGGCGTTC |
| 35 | 2350       | 2360        | 2370        | 2380       | 2390        | 2400       |
|    | AAGGAGCAGG | TGGAGGAGGC  | GCTGGCCAAA  | GCCGCCGAAA | AGAATCCCGA  | GGCGGGTAAG |
| 40 | 2410       | 2420        | 2430        | 2440       | 2450        | 2460       |
|    | TCGCTCGATT | CGCGGCAGGC  | GATCGTCGAG  | CGCAGCCTCA | CCGATACGTT  | GCTCGCCGGG |
| 45 | 2470       | 2480        | 2490        | 2500       | 2510        | 2520       |
|    | CTGGTGGTCG | AGCCGATCCT  | CAACTCGCGC  | CTGGTGTACG | TCAATTACGA  | TTCGCCAGAC |
| 50 | 2530       | 2540        | 2550        | 2560       | 2570        | 2580       |
|    | CCGGTGCTGG | CCGCCAAGAT  | CGCCAATACG  | TACCCGAAGG | TGTTTCATCGT | CAGCACCCAG |
| 55 | 2590       | 2600        | 2610        | 2620       | 2630        | 2640       |
|    | GAACGCCGCA | TGAAGGCGTC  | TTCGTTTGCG  | ACACAGTTTC | TGGCTGAGCG  | CCTGAAGCAG |
|    | 2650       | 2660        | 2670        | 2680       | 2690        | 2700       |
|    | TTGCGCGAGA | AGGTCTGAAGA | CTCTGAAAAG  | GATCTGGTCT | CGTATTCGAC  | CGAAGAGCAG |

2710 2720 2730 2740 2750 2760  
 ATCGTGTCCG TTGGCGATGA CAAGCCCTCG CTGCCTGCGC AGAATCTGAC CGATCTCAAT  
 5  
 2770 2780 2790 2800 2810 2820  
 GCGTTGCTGG CATCCGCACA GGACGCCCCG ATCAAGGCCG AGTCAGCTTG GCGGCAGGCT  
 10  
 2830 2840 2850 2860 2870 2880  
 TCCAGTGGCG ATGGCATGTC ATTGCCGCAG GTGTTGAGCA GCCCGCTGAT TCAAAGCCTG  
 15  
 2890 2900 2910 2920 2930 2940  
 CGCAGCGAGC AGGTGCGTCT GACCAGCGAG TACCAGCAGA AACTGTGCGAC CTTCAAGCCG  
 20  
 2950 2960 2970 2980 2990 3000  
 GATTACCCGG AGATGCAGCG CCTCAAGGCG CAGATCGAAG AGTCGCGTCG TCAGATCAAT  
 25  
 3010 3020 3030 3040 3050 3060  
 GGCGAAGTCA TCAATATCCG TCAGTCGCTG AAGGCGACCT ACGACGCCTC CGTGCATCAG  
 30  
 3070 3080 3090 3100 3110 3120  
 GAGCAGCTGC TCAACGACCG CATCGCCGGT CTGCGGTCCA ACGAGCTGGA TCTGCAGAGC  
 35  
 3130 3140 3150 3160 3170 3180  
 CGCAGCATCC GCTACAACAT GCTCAAGCGC GAACGTCGAC ACCAACC GCCC AGCTCTACGA  
 40  
 3190 3200 3210 3220 3230 3240  
 TAGCGCTCCT GCAGCGCTAC AAGGAAATCG GCGTGGCGAG CAACGTGGGC GCCAACAACG  
 45  
 3250 3260 3270 3280 3290 3300  
 TGACCATCGT CGATACCGCA GACGTGCCTA CGTCTAAGAC TTCGCCGAAA CTCAAATTGA  
 50  
 3310 3320 3330 3340 3350 3360  
 ACCTCGCGTT GGGCCTGATC TTTGGCGTAT TCCTGGGCGT GGCTGTGGCT CTGGTTCGCT  
 55  
 3370 3380 3390 3400 3410 3420  
 ACTTCCTGCG TGGGCCTTCT CCGAAGTCGC GGTGGAAGT ACATCGTGAT GTTGCAAAAC  
 3430 3440 3450 3460

GATGGTTAAT TGAAGTGACA ACTGATTCAG CGTGGAAAAG GTGGGATCC

The sequence of the 2.2 Kb BamHI fragment is as follows:

|    |            |            |            |            |             |            |
|----|------------|------------|------------|------------|-------------|------------|
| 5  | 10         | 20         | 30         | 40         | 50          | 60         |
|    | GGATCCAGCT | TGTATGTGCT | CCCGGCCTTG | TGGTTTCTCC | CCGCACTGTT  | TGTCGCCACC |
| 10 | 70         | 80         | 90         | 100        | 110         | 120        |
|    | GTTGTCTACC | TGGCACTGCG | CGAAGACCTG | AGCGCGCAGT | GCTCGCGGTC  | TGCAGTTTGC |
| 15 | 130        | 140        | 150        | 160        | 170         | 180        |
|    | TGGTTGTGTG | GGCGTGGACG | CGTTGGTTCC | CAGGGCTGCG | GCTGCGCTTC  | CGTTTGCAC  |
| 20 | 190        | 200        | 210        | 220        | 230         | 240        |
|    | GGATGTGCTG | CCGGTCGCGC | TGTTCTTCAT | TGCAGTCGGC | GCATGGCTGT  | CACGCTTCGC |
| 25 | 250        | 260        | 270        | 280        | 290         | 300        |
|    | AGAGAGAGTG | CGCGCGCTTC | CTGCGGTCGT | TTGGGTCGTC | GCGTTCCC    | GCATTC     |
| 30 | 310        | 320        | 330        | 340        | 350         | 360        |
|    | GCCTGGGGGG | GCGTTGCAGC | CATGAACGGG | CAGGTGGATG | TCAATAATCT  | TCAGTTCGGA |
| 35 | 370        | 380        | 390        | 400        | 410         | 420        |
|    | AAATCGTCGC | TCCTGTTTCT | GATCGCAAGC | CTGCTGGGTA | CAGCAATGAC  | GTTGTGCATT |
| 40 | 430        | 440        | 450        | 460        | 470         | 480        |
|    | GCCTACTTCA | TGCAAGGGTG | GCGCTGGCTG | CGTTGGATCG | GCGCCAATAC  | GCTGCTGATC |
| 45 | 490        | 500        | 510        | 520        | 530         | 540        |
|    | CTTGGCACGC | ACACGTTGGT | GTTTCTGGTC | GTGACCAGTG | TCGTGGTGCG  | AACCGGGGTG |
| 50 | 550        | 560        | 570        | 580        | 590         | 600        |
|    | ATCGATCGCA | AACTCATCGG | TACACCTGTC | TGGGCGCTGG | CTCTCTGCGC  | CTTTGCCATC |
| 55 | 610        | 620        | 630        | 640        | 650         | 660        |
|    | GCTGCCTGCA | TTCCCATGCG | TGCCGTGCTG | GTGCGCCGCG | CCCTGGATGT  | TGGGATTGAA |
|    | 670        | 680        | 690        | 700        | 710         | 720        |
|    | ACGCAAGTGA | GACATTTTCA | GAATCATCAG | TCGATGTGGC | GTGTTTCGTGT | GAGTCACCGG |

|    |             |            |            |             |            |            |
|----|-------------|------------|------------|-------------|------------|------------|
|    | 730         | 740        | 750        | 760         | 770        | 780        |
|    | CAAAGGAGAT  | CGGCGCAATG | AAAGTCGTGC | ATGTGGTCCG  | CCAGTTCCAT | CCGTCGATCG |
| 5  | 790         | 800        | 810        | 820         | 830        | 840        |
|    | GGGGGATGGA  | GGAAGTCGTG | CTGAACGTGG | CACGTCAGCA  | TCAGGCCAAC | AGTGCCGACA |
| 10 | 850         | 860        | 870        | 880         | 890        | 900        |
|    | CGGTTGAGAT  | CGTGACGTTG | GATCGTGTGT | TCACCGATCC  | CTTCGCGCAA | CTGGCGCAGC |
|    | 910         | 920        | 930        | 940         | 950        | 960        |
| 15 | ACGAGGTCCA  | TCAGGGGTTG | TCGATCACTC | GCATCGGCTA  | TCGTGGTTCA | TCGCGGTACC |
|    | 970         | 980        | 990        | 1000        | 1010       | 1020       |
| 20 | CGATCGCGCC  | GTCGGTGCTG | GGGGCGATCC | GTTCCGGCGGA | CGTGGTGCAT | CTGCATGGCA |
|    | 1030        | 1040       | 1050       | 1060        | 1070       | 1080       |
|    | TTGATTTTTT  | CTACGACTAC | CTGGCGTTGA | CCAAGCCGCT  | GCACGGCAAG | CCGATGGTGG |
| 25 | 1090        | 1100       | 1110       | 1120        | 1130       | 1140       |
|    | TCTCGACGCA  | TGGCGGGTTT | TTCCACACTG | CCTATGCGTC  | GCGCATGAAG | CAGATCTGGT |
|    | 1150        | 1160       | 1170       | 1180        | 1190       | 1200       |
| 30 | TCCAGACGCT  | GACGCGTACT | TCTGCGCTGG | CCTATGCGCG  | TGTGATCGCC | ACTAGCGAGA |
|    | 1210        | 1220       | 1230       | 1240        | 1250       | 1260       |
| 35 | ATGACGGCGA  | TCTGTTCGCC | AAGGTGGTCG | CGCCGTCGCG  | CTTGCGGGTG | ATCGAGAACG |
|    | 1270        | 1280       | 1290       | 1300        | 1310       | 1320       |
| 40 | GTCGTCGACG  | TGGAGAAGTA | TGCAGGGCAG | GGCGCTCGAG  | CGCCGGGACG | GACCATGCTG |
|    | 1330        | 1340       | 1350       | 1360        | 1370       | 1380       |
|    | TATTTTCGGGC | GTTGGTCGGT | CAACAAGGGC | CTGATCGAAA  | CGCTTGAATT | GCTGCAGGCT |
| 45 | 1390        | 1400       | 1410       | 1420        | 1430       | 1440       |
|    | GCGCTCACGC  | GTGATCCGCA | GTGGCGGTTG | ATCATCGCCG  | GGCGCGAGTA | CGATTTGAAT |
| 50 | 1450        | 1460       | 1470       | 1480        | 1490       | 1500       |

GAGGCGGATC TGC GCAAGGC CATCGCGAAC GCGGTTTGCA GGACAAGGTG CAGCTGAGCA  
 5           1510           1520           1530           1540           1550           1560  
 TGTCGCCATC GCAGCAGCAG TTGTGCGCGT TGATGCAGCA GGC GCAGTTC TTCGTGTGCC  
 10           1570           1580           1590           1600           1610           1620  
 TGTCGCGGCA TGAGGGGTTT GGGATTGCGG CGGTGGAAGC GATGAGCGCG GGGTTGATCC  
 15           1630           1640           1650           1660           1670           1680  
 CGATTCTCAG CGACATTCCT CCGTTCGTGC GGCTTGCCAC CGAGTCCGGA CAGGGTGTGA  
 20           1690           1700           1710           1720           1730           1740  
 TCGTCAATCG CGACAGGATT CAGGCCGCGG CCGACAGCGT GCAAGCATTG GCGCTGCAGG  
 25           1750           1760           1770           1780           1790           1800  
 CCAATGCGGA TTTCGATGCG CGCCGCACGG CGACCATGGC GTATGTGGCG CGCTACGACT  
 30           1810           1820           1830           1840           1850           1860  
 GGCGGCACGT GGTGGGGCGT TATATCGACG AGTACCACGT GCGCTGGGAA CACCACGTAC  
 35           1870           1880           1890           1900           1910           1920  
 GCAGGAGGCC GTGCGATGAG CGCGTCTGCT TCGCTGCCAG TGACGCGTGC TGCTGCGGCG  
 40           1930           1940           1950           1960           1970           1980  
 CCCC GGATCA CGGTGCTGTT CTCCACCGAA AAGCCGAACG CCAACACCAA CCCGTATCTC  
 45           1990           2000           2010           2020           2030           2040  
 ACCCAGCTCT ACGATCGCTG CCGGAGCGGT GCAGCCGCGC TTCTTTTCGA TGC GCGAGGC  
 50           2050           2060           2070           2080           2090           2100  
 GTTGTGTGTC CGCTACGACG TGCTGCATCT GCACTGGCCG GAATATCTGC TGC GCCATCC  
 55           2110           2120           2130           2140           2150           2160  
 CAGCAAGATG GGCACGCTGG CCAAGCAGGC CTGCGCTGCC TTGCTGCTGA TGAAGTTGCA  
 60           2170           2180           2190           2200           2210           2220  
 GCTGACCGGC ACGCCGGTGG TACGCACCTT GCACAACCTG GCGCCGCATG AAGACCGCGG

2230                      2240                      2250                      2260                      2270  
 CTGGCGGAGC GCGCGCTGCT GCGTGGATCG ATCAGCTCAC GCGGCGCTGG ATCC

CGCCGGTGG TACGCACCTT GCACAACCTG GCGCCGCATG AAGACCGCGG

5  
 10 The G+C content of *Xanthomonas* DNA is relatively high (reported in Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984) Williams and Wilkins, Baltimore, Maryland, as 65-70 percent). Because of this high G+C content, band compressions on the sequencing gels occur at a relatively high frequency. This problem has been addressed in two ways: (1) the nucleotide sequence is derived for both strands of the DNA so that all sequence can be verified by complementarity of strands and (2) deoxyinosine was substituted for deoxyguanosine to obtain the sequence in areas where band compression was an acute  
 15 problem. In spite of these measures to avoid errors, the sequence shown above is subject to slight uncertainty due primarily to technical difficulties resulting from the high G+C content of the DNA.

#### Example 10

20 This example describes cloning a large segment of *X. campestris* DNA that contains all of the DNA known to encode gum biosynthetic genes.

In order to efficiently transfer the gum gene cluster from *X. campestris* to alternative production organisms, the gum gene cluster was cloned onto plasmid pRK290, described by Titta *et al.* Proc. Natl. Acad. Sci. USA 77:7347-7351 (1980), specifically incorporated herein by reference. This plasmid vector has  
 25 a low copy number, a broad host range, and can be conjugally transferred from *E. coli* to a wide variety of gram-negative bacteria. It was found that the restriction enzyme *DraI* does not cut within any of the cloned *X. campestris* DNA of the gum region, however it does cut several times within the lambda cloning vector used in construction of the *X. campestris* gene bank. Cleavage of recombinant bacteriophage DNA with *DraI*, therefore, generated several DNA fragments, one of which contains the entire *X. campestris* insert and  
 30 a relatively small amount of lambda DNA (approximately 3 kb) fused on each end. Therefore, it was decided to choose a lambda recombinant that contains all the *X. campestris* DNA believed to contain gum genes, to purify a large quantity of that phage DNA, to digest the DNA with *DraI*, and to purify and subsequently clone the restriction fragment that contains the *X. campestris* insert DNA.

Upon isolation of each lambda phage carrying gum gene DNA, a *Bam*HI restriction map was  
 35 constructed for each phage. As a result of the mutational analysis, a picture developed of which segments of cloned DNA carried gum genes. It was concluded that lambda 655(L'), shown in Figure 2, contained the intact gum gene cluster. Therefore, this phage was chosen for initial cloning efforts. Using standard techniques, a large-scale (1 liter) lysate of lambda 655(L') was prepared. This lysate yielded a total of approximately  $8 \times 10^{11}$  phage particles. The phage particles were purified by polyethylene glycol precipitation and CsCl density gradient centrifugation. Phage DNA was extracted from the virions, and a yield of  
 40 approximately 225 ug was obtained.

Approximately half of the purified lambda 655(L') DNA (120 ug) was treated with *Eco*RI methylase and *Bam*HI methylase. These enzymes recognize and modify, i.e., methylate, the nucleotide sequences recognized and cleaved by the corresponding restriction endonucleases. Methylation renders the sequence  
 45 resistant to cleavage by the endonuclease. Methylation allows use of *Bam*HI or *Eco*RI oligonucleotide linkers on the ends of the *DraI* fragments, which are blunt and therefore poor substrates in ligation reactions. The success of the methylation reaction is monitored by analyzing subsequent resistance of lambda 655(L') DNA to cleavage by *Eco*RI and *Bam*HI. The methylated DNA was not perceptibly cleaved by an approximately 20x excess of each enzyme in a 2-hour digestion.

50 Roughly 100 ug of methylated DNA was then digested to completion with *DraI*, and approximately 80 ug of this DNA was then reacted in a ligation with an 8 bp *Bam*HI oligonucleotide linker. The linker is a small double-stranded, blunt-ended DNA which is ligated onto the blunt *DraI* ends in a reaction using high DNA concentrations and approximately 100x molar excess of linker fragment over *DraI* lambda 655(L') fragment. The ligated linker fragments can subsequently be cleaved with *Bam*HI to generate sticky ends  
 55 suitable for cloning into *Bam*HI-cut vector DNA. However, prior to *Bam*HI digestion, the DNA is fractionated by sedimentation through a sucrose gradient. The purpose of fractionation over sucrose is two-fold. First, a large enrichment of the 20 kb fragment can be achieved, although the resolution of sucrose gradient sedimentation will not afford absolute purification of the 20 kb fragment away from the other *DraI* fragments.

Second, the unligated BamHI linker molecules can be eliminated, because these small DNA segments essentially do not enter the gradient. Thus, the gradient fractions containing the 20 kb DraI fragment will be, for practical purposes, completely free of linker DNA. This is important because the linker DNA can potentially interfere with subsequent digestion and/or ligation steps. The DraI digest was centrifuged through  
 5 a 5 ml 5% to 20% sucrose gradient. Fractions (approximately 250  $\mu$ l) were collected and analyzed by running an aliquot of each fraction on an agarose gel. Fractions containing the bulk of the 20 kb DraI fragment were pooled, ethanol precipitated, resuspended in buffer, and stored for future use.

The 20 kb DraI fragment was then digested with BamHI in order to cleave the attached linker molecules and generate single-stranded DNA ends suitable for cloning. The plasmid pRK290 was digested with BglII,  
 10 which generates single-stranded DNA ends identical to the BamHI ends. The ligation reaction was carried out in 50 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 20 mM dithiothreitol, and 1.0 mM ATP. The 20  $\mu$ l reaction contained 0.2  $\mu$ g of pRK290 (digested with BglII) and 0.1  $\mu$ g of 20 kb DraI fragment (digested with BamHI) and was catalyzed by T4 DNA ligase (6 Weiss units). The reaction was allowed to proceed overnight (approximately 18 hours) at 12°C.

The products of the ligation reaction were then used to transform E. coli. Tetracycline-resistant transformants were selected and then screened for the presence of recombinant plasmids containing the cloned gum gene cluster. This screening was accomplished by mating individual tetracycline-resistant transformants with a X. campestris strain containing a Gum<sup>-</sup> mutation (within the gum gene cluster), selecting for conjugal transfer of the tetracycline resistance into X. campestris and visually assessing the  
 20 resulting Gum phenotype. Most matings produced Gum<sup>-</sup> Tet<sup>r</sup> X. campestris, but a small number yielded Tet<sup>r</sup> colonies that appeared very mucoid. Analysis of plasmids carried by E. coli plasmid donors in these matings revealed the presence of recombinant plasmids comprised of pRK290 and the inserted gum gene cluster DNA.

Five recombinant plasmids have been analyzed. Purified plasmid DNA's were prepared by CsCl density  
 25 gradient centrifugation and analyzed by BamHI digestion and agarose gel electrophoresis. The restriction fragments generated were compared to the BamHI digestion products of lambda 655(L'). The vector pRK290 has no BamHI sites (the DraI fragment, carrying BamHI linkers on its ends, was cloned into a BglII site) and the lambda 655(L') phage contains no BamHI sites outside the cloned X. campestris DNA. Therefore, the BamHI digests should produce identical sets of X. campestris DNA fragments. However, only  
 30 two pRK290 derivatives (H627 and H806) contained all of the BamHI fragments found in lambda 655(L'). The other three recombinants each contained a unique and contiguous subset of these BamHI fragments. The most likely explanation for these truncated clones is that the methylation of BamHI sites (a step in preparation of the DraI fragment for cloning) was incomplete. That is, a small fraction of the BamHI sites were not methylated and therefore not protected against the subsequent BamHI digestion used to cleave  
 35 the attached linkers. If, for example, 5% of the BamHI sites were unprotected, then 40% of the DraI fragments would contain an unmethylated BamHI site. The subsequent BamHI digestion would cleave these sites and produce a family of truncated fragments that could be cloned in pRK290. The assay of BamHI methylation used to check the initial reaction would not have detected a 5% level of unmethylated BamHI sites. The incomplete clones probably arose as described above. In any event, the structures of the five  
 40 clones obtained are shown in Figure 9.

#### Example 11

This example shows, through the use of immunologic assays, that the enzymes encoded by the gum  
 45 biosynthetic cluster were present only in small quantities in X. campestris.

Proteins were displayed through the technique of two-dimensional electrophoresis. Wild-type X. campestris was compared to a X. campestris strain unable to synthesize a sugar nucleotide precursor, but which was able to synthesize xanthan *in vitro*. These two strains contain gum biosynthetic enzymes. The protein proposed to be UDP-glucose dehydrogenase was missing in the pattern of the second strain. An X.  
 50 campestris strain deleted for the entire gum biosynthetic cluster was also analyzed. Several proteins were missing from extracts of the deletion strain. The intensity of the spots proposed to correspond to the gum biosynthetic enzymes was very low.

Additional data are provided. An insertion mutation in the central portion of the 2.2 kb BamHI fragment produces a Gum<sup>-</sup> mutant which is defective in Transferase III activity. This insertion is located at the BglII  
 55 site approximately at position 1132 in the DNA sequence of the 2.2 kb BamHI fragment. The DNA sequence indicates that a large open reading frame (ORF) is present in this region of the sequence. The putative protein product having Transferase III activity begins with the ATG start codon at approximately position 738. Eight base pairs upstream from the start codon is a ribosome binding site (AGGAGA). The ORF clearly

extends well beyond the BglIII site where the insertion mutation defining the Transferase III activity is located.

Peptides corresponding to several regions of the predicted protein sequence were selected for their predicted immunogenicity by a hydrophilicity plot known to those in field and developed by Hopp and Woods and described in the article entitled "Prediction of Protein Antigenic Determinants from Amino Acid Sequences," in Proc. Natl. Acad. Sci. USA, Vol. 78, No. 6, pp. 3824-3828, June 1981, specifically incorporated herein by reference. The peptides chosen and their location within the 2.2 kb BamHI fragment are shown in Table 2.

Table 2

Peptides chosen from the 2.2 kb BamHI fragment for antisera production.

| Approximate location<br>in 2.2 kb<br>fragment (first bp) |    | Synthetic Peptides (13 residues) |     |     |     |     |     |     |  |
|----------------------------------------------------------|----|----------------------------------|-----|-----|-----|-----|-----|-----|--|
| 807                                                      | A. | Val                              | Ala | Arg | Gln | His | Gln | Ala |  |
|                                                          |    | Asn                              | Ser | Ala | Asp | Thr | Val |     |  |
| 930                                                      | B. | Arg                              | Ile | Gly | Tyr | Arg | Gly | Ser |  |
|                                                          |    | Ser                              | Arg | Tyr | Pro | Ile | Ala |     |  |
| 1041                                                     | C. | Leu                              | Ala | Leu | Thr | Lys | Pro | Leu |  |
|                                                          |    | His                              | Gly | Lys | Pro | Met | Val |     |  |
| 1632                                                     | D. | Asp                              | Ile | Pro | Pro | Phe | Val | Arg |  |
|                                                          |    | Leu                              | Ala | Thr | Glu | Ser | Gly |     |  |
| 1677                                                     | E. | Val                              | Ile | Val | Asn | Arg | Asp | Arg |  |
|                                                          |    | Ile                              | Gln | Ala | Ala | Ala | Asp |     |  |
| 1740                                                     | F. | Ala                              | Asn | Ala | Asp | Phe | Asp | Ala |  |
|                                                          |    | Arg                              | Arg | Thr | Ala | Thr | Met |     |  |

The peptides were synthesized by solid-phase peptide synthesis, conjugated to bovine serum albumin and used to immunize rabbits. After four immunizations, the hyperimmune sera were found to contain respectable titers of antibodies specific to the immunizing peptides by ELISA (enzyme-linked immunosorbent assay) widely used in the field and herein referenced Eva Engvall, Methods in Enzymology, Vol. 70, pp. 419-439, 1980, "Enzyme Immuno- Assay ELISA and EMIT".

These specific antibodies also reacted with a 40kd protein encoded by the 2.2 kb BamHI fragment on Western immunoblots as described by Towbin, H., Staehelin, T. and Gordon, J. in "Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications," Proc. Natl. Acad. Sci. USA 76:4350-4354 (1979), specifically incorporated herein by reference, of cell lysates of X. campestris S4-L. The protein was not present in cell lysates of X1107, the strain with the gum cluster DNA deleted, or X928, a strain with a mutational insertion in the 2.2 kb BamHI fragment. This 40 kd protein is not visualized in S4-L, by the less sensitive method of direct staining of protein in the gel with Coomassie blue; thus, this gum biosynthetic protein is a very small percentage of the total cell protein. It appears that low expression of the gum biosynthetic proteins is sufficient for xanthan gum production in S4-L.

Example 12

This example discusses the specific sugar nucleotide pools identified in various X. campestris strains that are Gum<sup>-</sup> in vivo, and in bacteria contemplated as alternative hosts.

5 Several Gum<sup>-</sup> strains were able to make xanthan in vitro when supplied with the sugar nucleotides UDP-glucose, GDP-mannose, and UDP-glucuronic acid. These mutants were defective in sugar nucleotide synthesis, rather than biosynthesis of xanthan itself. Subsequently, such mutants were found to be sensitive to the dye toluidine blue. Additional sugar nucleotide mutants were obtained by screening other Gum<sup>-</sup> strains for toluidine blue sensitivity.

10 The following method was employed to identify the specific sugar nucleotide defects in strains which were Gum<sup>+</sup> in vitro but Gum<sup>-</sup> in vivo. An isolated colony from each strain was picked and inoculated into 10 ml YM broth (3 g yeast extract, 3 g malt extract and 5 g peptone per liter) with 2% glucose in a 125 ml Erlenmeyer flask. Cultures were incubated at 30 °C, 250 rpm, for 24 hours or until turbid. Five percent inocula were transferred from YMG broth into mineral salts medium with glucose as the carbon source and  
15 allowed to grow at 30 °C for 24 hours. Cultures were harvested by centrifugation, washed twice with salts, and resuspended in a volume to sufficient to give an absorbance at 600 nm of 100. Samples, 1.5 ml, were placed in 50 ml Erlenmeyer flasks containing sufficient glucose to bring the concentration to 20mM. Each sample was incubated in a 30 °C water bath at 400 rpm for ten minutes, then 1 ml was removed and added to 0.1 ml of 11 N formic acid in an Eppendorf centrifuge tube. The tube was capped, the contents mixed for  
20 5 seconds on a vortex mixer, then the tube was placed in a dry ice-alcohol bath. After all samples had been processed, the tubes were removed from the dry ice bath, thawed at room temperature, and centrifuged for 5 minutes to pellet the cell debris. The supernatants were placed in prechilled 15 ml conical centrifuge tubes and frozen in a dry ice-alcohol bath again. Frozen samples were placed on a lyophilizer and taken to dryness. The contents of each tube were dissolved in 0.2 ml HPLC buffer, 40 mM phosphoric acid adjusted  
25 to pH 6.5 with triethylamine (Aldrich). Samples were filtered through 0.45 µm filters into microsample vials, then analyzed by injection onto a 4.6 mm x 250 mm C18 reverse phase ion pair column, 40 °C, flow rate of 0.8 ml per minute. Sugar nucleotides were identified by comparing retention times to those of standards run under the same conditions, and by examining the spectra of compounds eluting in the region of interest.

Four classes of sugar nucleotide mutants of X. campestris were identified using this procedure:

- 30 (1) mutants unable to synthesize UDP-glucose and UDP-glucuronic acid;  
(2) mutants unable to synthesize GDP-mannose;  
(3) mutants able to synthesize UDP-glucose but not UDP-glucuronic acid; and  
(4) mutants unable to synthesize UDP-glucose, GDP-mannose, and UDP-glucuronic acid.

35 Extracts from wild-type strains of X. campestris had all of the sugar nucleotides required by xanthan biosynthesis. Gum<sup>-</sup> mutants defective in the xanthan biosynthetic pathway itself had higher concentrations of the precursor sugar nucleotides than did wild-type cells. These data indicate that the rate of xanthan synthesis in wild-type cultures may be limited by the supply of precursor sugar nucleotides.

The sugar nucleotides in Paracoccus denitrificans (ATCC 17741), Pseudomonas stutzeri (ATCC 17588), and Pseudomonas perfectomarina (ATCC 14405) were analyzed using procedures developed for X.  
40 campestris. All organisms were grown for twelve hours in DENITE medium, a mineral salts medium, with two percent glucose as the carbon source. Cells were collected, washed, and resuspended to an absorbance at 600 nm of 100. The cell pellets had a pink hue typical of denitrifying bacteria which have derepressed synthesis of the cytochromes required for anaerobic growth (a typical response to oxygen limitation during growth). Consequently, 25 mM nitrate was included as an additional electron acceptor in  
45 the incubation mixtures. Cell suspensions were incubated with 20 mM glucose for five minutes with and without nitrate, then extracted with formic acid. Extracts were lyophilized, dissolved in TEA-phosphate buffer and analyzed by HPLC. Paracoccus denitrificans had UDP-glucose and GDP-mannose, but undetectable amounts of UDP-glucuronic acid, as verified by spectra of peaks in the regions of interest. Similarly, Pseudomonas perfectomarina and Pseudomonas stutzeri had UDP-glucose and GDP-mannose. UDP-  
50 glucuronic acid was not detected in extracts from either organism.

Example 13

This example shows that several gum biosynthetic enzymes have been expressed in the alternative  
55 host E. coli.

Many gum cluster DNA fragments have been cloned in the expression vector pp3, which was engineered from the plasmid pKO-1. That plasmid was described by K. McKenny et al. in Gene Amplification and Analysis, Volume II Elsevier, North Holland, p. 383, 1981, specifically incorporated herein

by reference.

The 2.2 kb BamHI fragment of the gum cluster encoding Transferase III was cloned into pp3, resulting in pJP1. E. coli JM105 transformed with pJP1 was cultured in rich medium, induced with IPTG ( $10^{-3}$  M) and harvested by centrifugation after 3 hours at 37°C. A cell lysate was prepared by two passes through a French pressure cell at 18,000 psi and run on a 10% SDS-acrylamide gel to separate the proteins by molecular weight. These proteins were probed with antibodies specific to Transferase III by the Western immunoblot technique. The cell lysate of JM105(pJP1) showed a distinct band of the 40 kd protein. The similarly-treated E. coli cell lysate with pp3 without an insert did not have the 40 kd protein. Even in the JM105(pJP1) cells induced by IPTG, the 40 kd protein from the X. campestris cloned DNA was not visible by Coomassie blue staining and thus was a small percentage of the total cell protein. When the electroblots of E. coli JM105 (pJP1) were probed with affinity-purified antibodies, the antibodies reacted singularly with Transferase III. The gum biosynthetic gene was unequivocally expressed in E. coli JM105.

Further evidence is given by the expression of fragments of the gum biosynthetic DNA cluster cloned into pp3 which were used to transform E. coli FD1098 (F'lacI<sup>Q</sup>). FD1098 is a strain that gives off, by an unequal cell division, minicells which do not contain chromosomal DNA but do contain copies of the pp3-derived plasmids. The use of minicells to analyze gene expression is described by J. E. Clark-Curtiss and R. Curtiss III in *Methods in Enzymology* 101:347-362 (1983), specifically incorporated herein by reference. After the minicells have been separated from whole cells by centrifugation through a series of sucrose gradients, the minicells are induced with IPTG and they are radiolabeled while expressing proteins encoded by the pp3-derived plasmid. The minicells are harvested, run out on 10% SDS-acrylamide gels, and the expressed proteins are visualized by autoradiography.

The gum biosynthetic DNA clearly encoded several proteins that were visualized by this method. A protein of 40 kd was again seen in E. coli, and it was dependent on the presence of the 2.2 kb BamHI fragment. A protein of 47 kd molecular weight was encoded by the 3.5 kb BamHI fragment. A protein of 27 kd is encoded by the X. campestris DNA spanning the 3.5 and the 1.35 kb BamHI fragments. The evidence for other gum biosynthetic proteins in E. coli minicells is tentative. The expression of gum cluster DNA is low in the alternate host E. coli, as is true in X. campestris.

#### Example 14

This example contemplates the means for achieving gene expression in an alternative host.

It is conceivable that the gum cluster of biosynthetic genes, when introduced into an alternative host, will be either transcriptionally silent or translationally silent. It is conceivable also that some, but not all, of the biosynthetic genes will be expressed. With nucleic acid probes and antibodies directed against the proteins encoded by the gum cluster, transcription and translation of all genes within the cluster will be measured.

If some or all of the genes are not transcribed, regulatable promoters will be added to the cluster in appropriate locations. The RNA polymerases of the eubacteria are very similar, including those from Gram-negative and Gram-positive species. The DNA sequences that permit initiation of transcription are sufficiently understood for one familiar with the art to introduce such promoters with ease.

Similarly, both Gram-positive and Gram-negative ribosome binding sites (which are used to initiate translation) are understood sufficiently to achieve translation of any mRNA which has been transcribed. Using standard methods of site-directed mutagenesis, any gum cluster enzyme that is inappropriately expressed relative to its expression in wild-type X. campestris will be adjusted for correct output in the alternative host.

#### Example 15

This example describes increased thermo-stability of gum biosynthetic enzymes.

The present invention contemplates the use of organisms that grow above 30°C as potential alternative hosts. Xanthan biosynthesis occurs optimally in X. campestris at temperatures between 27°C and 30°C. If the enzymes involved in gum biosynthesis do not function well above 30°C but are expressed, the enzymes will be altered to thermo-stable variants as described in Liao et al. in United States Patent Application Serial No. 793,475 entitled "Method for the Generation and Detection of Enzyme Variants with Enhanced Thermostability and Activity," filed October 28, 1985 as a continuation of United States Patent Application Serial No. 532,765, filed September 16, 1983, specifically incorporated herein by reference. Single amino acid substitutions in a protein can raise the maximum temperature at which an enzyme functions by more than 10°C. The gene sequences described herein readily permit such replacements.

Example 16

This example describes additional improvements that will allow alternative hosts to synthesize polysaccharides.

- 5 Alternative hosts containing the gum biosynthetic genes, and capable of transcription and translation of those genes, may fail to produce polysaccharide at all or at a significant rate.

The first experiments, if this occurs, will address the abundance and potential synthetic rate of the sugar nucleotide precursors (UDP-glucose, UDP-glucuronic acid and GDP-mannose). Cloned genes for the key enzymes of the sugar nucleotide pathways are available from X. campestris using methods described  
10 by Betlach et al., supra. If a particular sugar nucleotide pool is too low for adequate polysaccharide production, the appropriate X. campestris genes will be added to the alternative host. The alternative host may then yield adequate polysaccharide, or the rate may remain low.

If the rate of polysaccharide synthesis remains low, the second improvement will be attempted by surveying all other regions of the X. campestris genome for genes that allow the gum gene cluster and, if  
15 required, the sugar nucleotide pathway, to function so as to produce polysaccharide. A library of X. campestris genomic fragments as constructed in Example 3 of ca. 20,000 base pairs in length will be conjugally added by standard techniques as described by Titta et al. to the alternative hosts that have all of the gum cluster genes and sugar nucleotide genes but are still not making polysaccharide. Recipients of this library will be observed on petri plates for mucoid colonies; bacterial colonies that produce polysaccharide are easily distinguished from those that do not. Even when crowded, petri plates with  $10^3$  bacterial colonies are easily observed. Only a small number of recipients must be observed (less than  $10^3$ ) to see whether any other gene cluster present on an X. campestris DNA fragment will provide a missing gene product required for polysaccharide production.

If this experiment fails to yield a polysaccharide producer, the entire collection of recipient alternative  
25 hosts (now containing the well-expressed gum biosynthetic genes, the missing sugar nucleotide enzymes, if necessary, and a random collection of 20,000 base pair DNA fragments from X. campestris) will be mutagenized with chemicals that cause base-pair substitutions (such as, but not exclusively, nitrosoguanidine, 2-aminopurine or ethylmethane sulfonate). Mutagenized bacteria will be plated on agar medium so as to observe mucoid colonies. Mucoid colonies have been found as very rare revertants of  
30 some transposition-induced Gum<sup>-</sup> mutations of X. campestris. More than  $10^8$  mutagenized colonies of any given alternative host will be screened for polysaccharide production.

The choices of alternative hosts need not be restricted to a few best candidates. Plasmid pRK290-H336 has a broad host range and can be conjugally moved to a large number of potential alternative production strains. Similar constructs carrying the sugar nucleotide biosynthetic enzyme(s) will be made so that many  
35 alternative hosts can be tried. Thus far, pRK290-H336 has been placed within the potential production strains Pseudomonas putids, Pseudomonas cepacis, Pseudomonas denitrificans, Pseudomonas fluorescens, Pseudomonas stutzeri, Escherichia coli, and Enterobacter cloacae.

One strategy for alternative host selection would be to select a bacterium that is known to be capable of extracellular polysaccharide biosynthesis. Such strains can easily be mutated to incapacitate the en-  
40 dogeneous polysaccharide biosynthesis before pRK290-H336 is incorporated into the strain. Such strains could contain important gene products that must interact with the X. campestris gum biosynthetic enzymes and/or biosynthetic intermediates in order to facilitate polysaccharide synthesis and secretion. Since the plasmid transfer experiments are so straightforward, hosts with widely varying capacities to make a gum will be tried.

The proposed invention contemplates an alternative host, producing polysaccharide, within which reside the gum gene cluster of biosynthetic enzymes, the genes which provide for the biosynthesis of the appropriate sugar nucleotides and a random X. campestris DNA fragment. In addition, the strain may contain a number of base pair substitution mutations from the last step of the strain improvement. The alternative host will be successively cured, by standard techniques, of the random X. campestris DNA  
50 fragment and the sugar nucleotide biosynthetic gene. If polysaccharide production remains unchanged, these DNA fragments will not be used in the alternative host production strains.

Finally, the successful alternative host will be used in combination with those mutations in the gum biosynthetic cluster that cause synthesis of variant polysaccharides. Thus, the alternative host, with its combination of advantages from metabolic rate, growth temperature, and anaerobic metabolism, will be  
55 used to make xanthan and the xanthan variants of Vanderslice et al. and Doherty et al. and any other exopolysaccharide.

**Example 17**

This example describes the derivation of the nucleotide sequence for a 16 kb stretch of *Xanthomonas* genomic DNA which contains a cluster of genes involved in xanthan gum biosynthesis. A portion of the DNA sequence (i.e., the sequence of the 0.3, 1.4, 1.5, and 2.2 kb BamHI fragments) that is presented here was previously described in the patent application of Capage et al. entitled "Recombinant-DNA Mediated Production of Xanthan Gum" filed March 26, 1986. The previous sequence has been modified (corrected) by more recent results which were derived from sequencing reactions that utilized deoxyinosine to resolve areas of band compression on the sequencing gels. This example also shows how appropriate analysis of the DNA sequence reveals the structure and organization of the gum genes.

The complete nucleotide sequence for the entire 16 kb segment of DNA is presented in a double-strand format in Figure 10. The top strand of the sequence reads 5' to 3' in the left-to-right direction of the BamHI restriction map shown in Figure 4. The computer technique (frame analysis) described by Bibb et al. in Gene 30:157-166 (1984), specifically incorporated herein by reference, was used to determine the G+C distribution at each of the three nucleotide positions that define the three possible reading frames of the sequence in each direction. These frame analysis results showed that all of this DNA is transcribed in the left-to-right direction. Thus, the top strand of the sequence shown in Figure 10 is the coding strand sequence (i.e., the sequence of the mRNA with T substituted for U). The exact location of the genes defined by the sequence can be obtained from data that is shown in Table 3.

Table 3  
Location and characteristics of the gene  
proteins within the DNA of the gum gene cluster

| Protein | BamHI Fragment | Location (BP number) | Reading Frame | Mutant Phenotype        | Function               | Molecular Wt. (kD) |
|---------|----------------|----------------------|---------------|-------------------------|------------------------|--------------------|
| gpa     | 0.3 and 1.4    | 33 669               | 3             | Gum <sup>+</sup>        | ---                    | 24.4               |
| gpb     | 1.4 and 1.5    | 1336 1975            | 1             | No charger (lethal)     | Not Known              | 23.3               |
| gpc     | 1.5            | 2050 3181            | 1             | No charger (lethal)     | Not Known              | 42.8               |
| gpd     | 4.7            | 3644 5096            | 2             | No charger              | Not Known              | 54.6               |
| gpe     | 4.7            | 5181 6477            | 3             | Lethal                  | Not Known              | 48.3               |
| gpf     | 4.7            | 6476 7568            | 2             | No acetylation          | Acetylase              | 39.9               |
| gpg     | 4.7 and 2.2    | 7567 8704            | 1             | Mucoid Gum <sup>+</sup> | Not Known              | 42.0               |
| gph     | 2.2            | 8703 9843            | 3             | Cellobiose              | Transferase III        | 42.1               |
| gpi     | 2.2 and 3.5    | 9842 10889           | 2             | No charger              | Putative Transferase I | 38.9               |
| gpj     | 3.5            | 10909 12382          | 1             | Possibly Lethal         | Not Known              | 52.9               |
| gpk     | 3.5            | 12764 13649          | 2             | Trimer                  | Transferase IV         | 32.4               |
| gpl     | 1.35           | 13693 14485          | 1             | No pyruvylation         | Ketalase               | 29.3               |
| gpm     | 1.35 and 1.0   | 14495 15284          | 2             | Glucose                 | Transferase II         | 28.6               |

An overview of the organization and structure of the genes contained in the 16 kb segment of DNA is presented in Figure 11. The top line in the figure is a BamHI restriction map and indicates the location of each of the BamHI restriction sites in the sequence shown in Figure 10. In Figure 11, the line drawn above the frame analysis curves shows the approximate position of some of the mutations that have been isolated and characterized (examples 1, 2, 7, 19, 20). The frame analysis curves presented in Figure 11 show the

distribution of G+C content at the first (blue line), second (red line), and third (black line) nucleotide positions. Note that the distribution of G+C content at the three nucleotide positions is non-random throughout most of the entire sequence, indicating that virtually all of this DNA codes for protein products. Each area of non-random G+C distribution along the sequence predicts regions of the DNA that code for protein products. The reading frame of each protein is defined by the nucleotide position having an intermediate value within each region of non-random G+C distribution. The points where the G+C distribution at the three nucleotide positions change predict either the beginning or end of a gene or the end of one gene and the beginning of the next. In each case, these points were found to correlate with the presence of either a start or stop codon in the appropriate reading frame.

Below the frame analysis curves, separate arrows are drawn to indicate the location and extent of each gene in the sequence. For convenience, each gene is designated with a letter, and that letter preceded by "gp" is used to designate its protein product. Above each arrow, the molecular weight of the protein product is shown in kD. Below each arrow, the name of each gene product is shown as its lettered name as well as its functional name for those cases where gene function could be derived from the mutant phenotype.

The frame analysis curves indicate that there were three areas of the sequence (centered at base numbers 900, 3400, and 12400) where the G+C content at each of the three nucleotide positions shows a random distribution. Hence, these three areas of the DNA were not expected to code for protein products. All three of these areas contain a transcription termination signal as defined by an area of sequence containing a run of T's that is preceded by a GC-rich region that forms a stem-loop secondary structure. The secondary structure of these terminators is shown in Figure 13. Based on the location of the transcription terminators, the DNA of the 16 kb segment is delimited into three transcription units. The arrows at the bottom of Figure 11 show the extent, location, and direction of transcription for each of the three units which are designated as transcription units I, II, and III.

The DNA sequence between each terminator and the beginning of the first gene within each transcription unit should contain a sequence (promoter) which specifies the point of transcription initiation. Since results reported in the literature indicate that the sequence of at least some *Pseudomonas* promoters bears a resemblance to the sequence of *E. coli* promoters, the appropriate regions of DNA sequence within each of the three transcription units were examined for homology to the consensus sequences of *E. coli* promoters at both the -10 (Pribnow Box) and -35 positions. It is generally believed that the sequence of the Pribnow Box is the most important of these two sequences in specifying the binding of RNA polymerase to the DNA to initiate transcription. The consensus sequences of the Pribnow Box and the -35 hexamer are TATAAT and TTGACA, respectively (capital letters designate the highly conserved nucleotides of each hexamer). Thus, a minimum criterion for defining a putative promoter is the presence of a hexamer having the sequence TANNNT ("N" is any nucleotide).

Transcription units I and III were found to contain putative promoters (at positions 1199 and 12664, respectively) that satisfied only the minimum criterion, i.e., homology with the first, second, and sixth nucleotides of the Pribnow Box. Thus, the location of the putative promoters for these two transcription units is very tentative. Transcription unit II contained a putative promoter (around position 3580) having a sequence bearing striking homology to the consensus sequence of *E. coli* promoters. The sequence of the Pribnow Box shows homology with four out of the six nucleotides, including the three that are highly conserved. The sequence of the -35 hexamer shows homology with five out of six nucleotides, including the four that are highly conserved. The distance between the two hexamers is 16 base pairs, in perfect agreement with the consensus distance of 16 to 19 base pairs. Thus, although it is not possible to unequivocally identify a promoter from sequencing data alone, it seems highly probable that the putative promoter for transcription unit II is correct.

The data presented in Figures 10, 11, and 12 and Table 3 (taken together) show a unified picture of the structure and organization of the gum genes as well as provide an easily accessible source of specific information. For example, Figure 11 shows that there is an insertion mutation in the 4.7 kb *Bam*HI fragment that generates mutant *Xanthomonas* cells that produce non-acetylated gum. The mutation is located around position 7000 of the DNA sequence in a region containing a frame 2 ORF that defines a protein product (gpF) having a molecular weight of 39.9 kD. The exact position of the gene within the DNA sequence is shown in Table 3. This information can be used to locate the DNA sequence of the gene in Figure 10. Figure 11 shows that the gene is located within transcription unit II and probably functions as the acetylase enzyme. The predicted amino acid sequence of the acetylase enzyme is shown in Figure 12 along with additional data that indicate that the enzyme contains a high proportion of hydrophobic amino acid residues that are distributed throughout the protein sequence. Therefore, the acetylase enzyme probably functions in the bacterial membranes.

The region of the DNA sequence which begins with the first nucleotide at the left-hand BamHI site of the 0.3 kb BamHI fragment and extends to the transcription terminator around position 800 is DNA that appears to lie outside of the region of the *Xanthomonas* chromosome that contains genes involved in gum biosynthesis. The DNA sequence in this area shows that there is a frame 3 ORF that spans the BamHI site that delimits the 0.3 and 1.4 kb BamHI fragments. The ORF defines a protein product (gpA) having a molecular weight of 24.4 kD. Insertions into the BamHI site that are located within the gene produce *Xanthomonas* cells with a Gum<sup>+</sup> phenotype. Thus, gpA appears to be the final gene in a transcription unit that begins somewhere to the left of the 0.3 kb BamHI fragment in a region of the *Xanthomonas* chromosome containing genes that are not involved in gum biosynthesis.

The region of DNA sequence between the end of gpA and the transcription terminator was found to contain a proline tRNA gene. The folded secondary structure of this tRNA transcript is shown in Figure 14. The G and C nucleotides at the top of the anticodon loop (shown circled in Figure 14) would probably preclude the functioning of this tRNA in translation. Chromosomal deletions which completely remove this tRNA gene as well as the entire gum gene cluster and insertions into the anticodon loop produce *Xanthomonas* cells that are viable but Gum<sup>-</sup> in the case of deletions and Gum<sup>+</sup> in the case of insertions. Thus, this proline tRNA gene is not essential for viability or gum biosynthesis.

The frame analysis curves (Figure 11) for the area of the DNA sequence contained within transcription unit I indicate that this region of the *Xanthomonas* chromosome contains a large frame 1 ORF. The location of start and stop codons (Table 3) shows that the ORF contains two proteins (gpB and gpC) having molecular weights of 23.3 and 42.8 kD, respectively. Initial results from studies done to characterize mutant *Xanthomonas* strains containing Tn10 insertions within the 1.4 and 1.5 kb BamHI fragments showed that both of these proteins exhibited a "no charger" mutational phenotype. However, these results were ambiguous since Southern blots of the chromosomal DNA from the mutant strains clearly indicated that the DNA had undergone a rearrangement. More recent results from complementation experiments showed that deletion of the 1.5 kb BamHI fragment was lethal in *Xanthomonas* cells. The *Xanthomonas* mutant X1231 contains a deletion of the entire 16 kb segment of DNA (which includes the 1.5 kb BamHI fragment) and, although the strain is Gum<sup>-</sup>, it remains fully viable. Thus, gpC or gpB or both are essential for viability in *Xanthomonas* cells which contain an otherwise functional gum gene pathway.

The DNA defining transcription unit II (Figure 11) is greater than 9 kb in length and codes for seven gene products. Insertion mutations in each of these genes have been isolated and characterized. The effects of insertions into the region of DNA defining gpJ are still uncertain. Preliminary results suggest that insertions in this region are probably lethal. Insertions at several places in the ORF defining gpG yield *Xanthomonas* cells that produced mucoid colonies which produce seemingly normal gum, although the quantity of gum produced may be somewhat reduced. The hydrophobic properties of the amino acids contained in gpG (Figure 12) indicate that this gene product is probably a membrane protein. We cannot explain the apparent absence of any deleterious effects from insertion mutagenesis. Two of the genes (gpF and gpH) in transcription unit II play an obvious role in gum biosynthesis. The mutational phenotypes for these two genes indicate that gpF is acetylase and gpH is transferase III. Two of the genes, gpD and gpl, show a "no charger" mutational phenotype. This phenotype would be expected for the gene defining transferase I as well as any gene product involved in the regulatory control of transcription and/or translation of the gum genes. This phenotype might also be expected for a gene product that plays a structural role in maintaining an enzyme complex that is necessary for gum biosynthesis. Three of the expected five genes that define the transferase enzymes have been clearly identified from their mutational phenotypes. These are gpH, gpK, and gpM, which are transferase III, IV, and II, respectively (see Figure 11). All three of these genes are composed of amino acids having a relatively low hydrophobicity profile (Figure 12) and are located in the DNA on the right-hand side of the gum gene cluster. If it is assumed that these properties are general characteristics of the *Xanthomonas* transferase enzymes, then gpl is clearly the best candidate to be transferase I, leaving gpD as a putative regulatory or structural protein that is necessary for gum biosynthesis. Insertions into the region of DNA occupied by gpE are lethal in *Xanthomonas* cells which contain an otherwise intact gum gene pathway. As was also the case for the genes located in the 1.5 kb BamHI fragment (discussed above), gpE itself is not an essential protein for cell viability since several deletion strains which remove the region of the chromosome containing gpE are Gum<sup>-</sup> but viable. It appears that gpE as well as gpC and/or gpB must be proteins that are necessary to prevent the accumulation of a product(s) (produced by the functioning gum genes) that is toxic unless it is further metabolized by the enzyme activities of gpE, gpC, and/or gpB. Thus, it seems likely that these proteins function in the polymerization and/or the transport of xanthan gum out of the cell.

The DNA following the transcription terminator at position 12570 and extending beyond the right-hand BamHI site of the 1.0 kb fragment (i.e., beyond the end of the sequenced DNA) defines what we believe is

a third transcriptional unit. It should be noted that the transcription terminator defining the end of the transcription unit II has a rather short hairpin stem with a free energy strength (G) of only -4.4 kcal/mole. If the strength of the hairpin is related to the efficiency of transcription termination, then it is possible that transcriptional read-through occurs in this area. Transcription unit III clearly contains at least three genes defined by mutational phenotypes which indicate that their protein products are transferase IV (gpK), ketalase (gpL), and transferase II (gpM). The transferase II gene terminates at position 15284 which is 227 base pairs in from the left-hand BamHI site of the 1.0 kb BamHI fragment. Beyond this point extending rightward to the end of the sequenced DNA, the frame analysis curves show a non-random G+C distribution at the three nucleotide positions that is characteristic of DNA that codes for a protein product. Although we were not able to clearly define the position, extent, or number of ORFs in this region, the frame analysis profile clearly indicates that this DNA contains at least one gene or possibly one gene and a small portion of a second gene, that spans the right-hand BamHI site of the 1.0 kb fragment extending into DNA that has not as yet been sequenced. On the other hand, insertions into the right-hand BamHI site of the 1.0 kb fragment as well as an insertion within the 1.0 kb fragment located about 300 base pairs to the right of the left-hand BamHI site both show a Gum<sup>+</sup> phenotype.

In general, membrane proteins contain a high percentage of hydrophobic amino acid residues (i.e., Phe, Trp, Tyr, Ile, Leu, Met, and Val). To determine which of the gum gene proteins were likely to be located in the bacterial membranes, the computer was used to determine the proportion of hydrophobic amino acid residues as well as the distribution of hydrophobic regions within each protein sequence. These data are presented in Figure 12 and show that proteins gpD, gpE, gpF, gpG, and gpJ contain a relatively high proportion of hydrophobic amino acid residues (greater than 40%) that are distributed throughout each amino acid sequence. Thus, these proteins are probably membrane proteins.

#### Example 18

This example shows that an enzyme encoded by the gum biosynthetic cluster is expressed in several alternate host strains of a different genus.

Antibodies which recognize Transferase III were purified by affinity chromatography and used to detect Transferase III on Western immunoblots. The production of the sera containing the antipeptide antibodies specific to the 40 kD protein identified as Transferase III is described in Example 11. One of these sera was absorbed to a peptide affinity column which consisted of the immunizing peptide which had been conjugated to CH-Sepharose via carbodiimide. The specific antipeptide antibodies were eluted from the affinity column with glycine-HCl (0.2 M, pH 2.6) and neutralized with Tris. The purified serum recognized specifically Transferase III on Western immunoblots (supra).

The affinity-purified antipeptide antiserum was used to detect the presence or absence of Transferase III in cell lysates of alternate host strains containing a plasmid with (pRK290-H336) or without (pRK290-H11) the gum cluster DNA (Example 16). The strains tested are shown in Table 4. The presence of Transferase III on the Western immunoblots indicated that the gum cluster DNA is being expressed in the alternate host strains. Transferase III was expressed in Pseudomonas denitrificans, Pseudomonas stutzeri, and Pseudomonas putida with pRK290-H336. The level of expression in Pseudomonas stutzeri was almost equivalent to that in Xanthomonas campestris itself. Expression of Transferase III off of pRK290-H336 in these alternative hosts is probably due to transcription of a messenger RNA that is initiated at an endogenous X. campestris promoter sequence located in the leftward end of the 4.7 kb BamHI fragment. The DNA sequence of the gum gene cluster, presented in Example 17, identifies two putative strong transcription terminators between Transferase III and the plasmid DNA of the vector pRK290. These terminator sequences, which occur at positions 814-844 and 3457-3493, would almost certainly prevent the transcription of Transferase III and any other downstream genes by RNA polymerase molecules that initiated transcription upstream of the terminators. Therefore, the transcript that contains the Transferase III gene must originate within the gum gene DNA and very likely originates from a putative promoter sequence located in the 4.7 kb BamHI fragment around position 3580. This argues that the transcription signals present in the gum cluster and normally used by X. campestris are also recognized and used in these alternative hosts. This implies that many or all of the gum genes carried on pRK290-H336 are transcribed and quite possibly translated in the alternative hosts.

The enzyme was not expressed from this plasmid construct in the E. coli strains tested. Evidently, the appropriate expression signals for E. coli must be present for the expression of xanthomonad DNA in E. coli. Example 13 showed expression of Transferase III from pp3, a plasmid with appropriate E. coli expression signals. It is expected that the other proteins encoded by the gum biosynthetic cluster can be expressed in a variety of alternate host strains.

Table 4

Expression of Transferase III in Alternate Host Strains

|    |                                                        | <u>Transferase III by<br/>Western Immunoblot</u> |
|----|--------------------------------------------------------|--------------------------------------------------|
| 5  |                                                        |                                                  |
|    | <u>Xanthomonas campestris</u> S4L                      | ++                                               |
| 10 |                                                        |                                                  |
|    | <u>Xanthomonas campestris</u> X928 (III <sup>-</sup> ) | -                                                |
|    | <u>Xanthomonas campestris</u> S4L (pRK290-H11)         | ++                                               |
| 15 |                                                        |                                                  |
|    | <u>Xanthomonas campestris</u> S4L (pRK290-H336)        | ++                                               |
|    | <u>Pseudomonas putida</u> (pRK290-H11)                 | -                                                |
| 20 |                                                        |                                                  |
|    | <u>Pseudomonas putida</u> (pRK290-H336)                | +                                                |
|    | <u>Pseudomonas fluorescens</u> (H11)                   | -                                                |
| 25 |                                                        |                                                  |
|    | <u>Pseudomonas fluorescens</u> (H336)                  | +                                                |
|    | <u>Pseudomonas stutzeri</u> (H11)                      | -                                                |
| 30 |                                                        |                                                  |
|    | <u>Pseudomonas stutzeri</u> (H336)                     | ++                                               |
|    | <u>Pseudomonas denitrificans</u> (H11)                 | -                                                |
| 35 |                                                        |                                                  |
|    | <u>Pseudomonas denitrificans</u> (H336)                | +                                                |
|    | <u>Pseudomonas cepacia</u> (H11)                       | -                                                |
| 40 |                                                        |                                                  |
|    | <u>Pseudomonas cepacia</u> (H336)                      | -                                                |
|    | <u>Enterobacter cloacae</u> (H11)                      | -                                                |
| 45 |                                                        |                                                  |
|    | <u>Enterobacter cloacae</u> (H336)                     | -                                                |
| 50 |                                                        |                                                  |
|    | <u>Escherichia coli</u> LE392 (H11)                    | -                                                |
| 55 |                                                        |                                                  |

Escherichia coli LE392 (H336) -

5 Escherichia coli SM32 (H11) -

Escherichia coli SM32 (H336) -

10

#### Example 19

This example describes methodology for regionally-directed mutagenesis of cloned gum gene DNA  
15 carried by recombinant plasmids pRK290-H336 and pRK290-HA3.

Regionally-directed mutagenesis was performed upon gum gene DNA carried by plasmids pRK290-H336 (H336) and pRK290-HA3 (HA3) described in Example 10. These cloned DNA segments were subjected to transposon mutagenesis in vivo and mutagenized in vitro through use of recombinant DNA technology to generate insertion and deletion mutations within the cloned X. campestris DNA. Plasmids  
20 carrying such mutations were transferred into X. campestris recipients including deletion mutant strains that lack all or nearly all of the gum gene DNA carried by H336. The resulting recipient strain carrying the mutant plasmid then immediately displays the gum phenotype that results from the plasmid mutation. This method eliminates the need for the gene replacement step utilized previously (Example 7).

Transposon mutagenesis employed a transposon described by Kleckner et al. in Gene 32:369-379  
25 (1984) and termed by her "element 12" and here referred to as TnK12 (Kleckner's element 12). This transposon carries a DNA segment containing the kanamycin resistance determinant of Tn5. In addition, this DNA segment also carries a gene encoding resistance to streptomycin. This gene does not function in E. coli but has been shown to be active in other gram-negative bacteria. Previously we demonstrated that this gene was active in Xanthomonas and conferred a streptomycin-resistant phenotype when introduced, on a  
30 plasmid, into Xanthomonas. Therefore, movement of TnK12 into Xanthomonas ought to similarly confer simultaneous resistance to kanamycin and streptomycin, thus affording a strong selection for plasmids carrying TnK12 insertions.

TnK12 is carried in the chromosome of an E. coli strain, NK7133, which is resistant to rifampicin, the drug we most frequently use to select for X. campestris recipients in plasmid transfer experiments.  
35 Therefore, we isolated and used a chloramphenicol-resistant (Cam<sup>r</sup>) derivative of the deletion strain X1107 (Figure 15) as the recipient in our initial TnK12 transposition experiments. The first experiment was performed as follows. The plasmid pRK290-H336 was introduced into strain NK7133 by transformation using purified plasmid DNA. We then performed a triparental mating using NK7133 (pRK290-H336) as the donor, LE392 (pRK2013) as a mobilizer, and X1107 Cam<sup>r</sup> as the recipient. Fresh overnight cultures of these  
40 strains were washed once in LB, and 3 ml of each parent were mixed together and filtered through a 0.45  $\mu$ m filter. The filter was incubated on an LB plate at 30°C for 4 hours. The cells were then resuspended off the filter in 3.0 ml (total) of YM broth. Aliquots were plated out on YMG plates containing chloramphenicol, kanamycin, and streptomycin or streptomycin alone. Each of 15 plates of each type of medium was spread with 0.1 ml of the resuspended mating mix. Plates were incubated at 30°C for 6-12 days and then scored.  
45 From this experiment, we ultimately obtained 100 Kan<sup>r</sup> Str<sup>r</sup> colonies. Most of these were Gum<sup>+</sup> and wild-type in appearance. Seven were Gum<sup>-</sup>, and five colonies were clearly mucoid but appeared to be morphologically distinct from wild-type Gum<sup>+</sup>. Fourteen of these Kan<sup>r</sup> Str<sup>r</sup> derivatives of X1107 were mated in triparental crosses with E. coli HB101 and X. campestris S4L rif-101 (R68.45 tet::Tn7). The plasmid R68.45 tet::Tn7 is a tetracycline-sensitive derivative of the conjugally active plasmid R68.45. In the mating,  
50 this plasmid serves as the mobilizer which directs transfer of pRK290-H336::TnK12 derivatives to E. coli. Because the plasmid gene encoding tetracycline resistance is inactivated (by virtue of the Tn7 insertion), selection for Tet<sup>r</sup> E. coli HB101 by selection for growth at 37°C on 10  $\mu$ g/ml of tetracycline results in specific selection for the transfer of the pRK290-H336 derivative into HB101. The analysis of the physical structure of these plasmids is technically easier in E. coli than Xanthomonas. The 14 matings all yielded  
55 Tet<sup>r</sup> derivatives of HB101 at frequencies ranging from 10<sup>-5</sup> to 10<sup>-4</sup> per recipient.

Plasmid DNAs were prepared from these stains and analyzed by restriction endonuclease digestions and agarose gel electrophoresis. Molecular weights of particular restriction fragments were determined by comparison of fragment mobility to the electrophoretic mobility of DNA fragment standards of known

molecular weight. The pattern of restriction fragments produced by particular enzymes allowed us to determine the positions of the TnK12 insertions. These are shown in Figure 15. Eleven insertions were found to be in the cloned gum gene DNA segment of pRK290-H336. All of these insertions resulted in a Gum<sup>-</sup> or mucoid phenotype when the particular mutant plasmid was present in X1107. Two Gum<sup>+</sup> plasmid derivatives were found to contain TnK12 insertions in the pRK290 portion of the molecule; this is consistent with the Gum<sup>+</sup> phenotype. One insertion (13) occurred in the vector but relatively near the gum gene DNA. This insertion conferred a slightly different Gum phenotype (morphologically), although the X1107 (pRK290-H336.13) strain produces large amounts of gum. This plasmid mutagenesis system allowed us to efficiently isolate and detect mutations within the cloned gum gene DNA. Using this procedure, with some minor variations, we isolated and characterized a set of TnK12 insertion mutations in pRK290-H336. In some experiments, a different *X. campestris* Gum<sup>-</sup> deletion strain was used. This deletion strain, X1231, (see Figure 15) is deleted for all of the gum gene DNA carried by pRK290-H336. Some experiments also used different selection schemes. For example, in certain instances kanamycin plus streptomycin or streptomycin alone were used to select for transfer of TnK12 from *E. coli* into *X. campestris*. Ultimately, 45 TnK12 insertions into pRK290-H336 were isolated and analyzed. Most of these were found to occur with the gum genes and most were simple insertions, although some did show evidence of secondary DNA rearrangements as well.

Insertion and deletion mutations have also been isolated in pRK290-H336 and pRK290-HA3 by *in vitro* mutagenesis using a 1.3 kb restriction fragment of transposon Tn903. This fragment can be excised from a plasmid (pUC4-K) by a variety of restriction enzymes, including *EcoRI*, *BamHI*, *Sall*, *AccI*, *HincII*, and *PstI*. The *HincII* digestion yields a DNA fragment with blunt ends which can be modified by the addition of a DNA "linker" molecule in order to generate DNA ends that can be ligated into other restriction sites. In general, the procedure for insertion mutagenesis with this fragment is analogous to the procedure used to isolate insertion and deletion mutations within cloned gum gene DNA carried in plasmid pMW79 as described in Example 7. The plasmid pUC4-K was digested by the appropriate restriction endonuclease and the 1.3kb Kan<sup>r</sup> fragment was subsequently purified from preparative agarose gels by electrophoretic elution out of gel slices. When it was necessary to add a DNA linker molecule to the end of the Kan<sup>r</sup> fragment, a *HincII* digestion of pUC4-K was ligated with the desired linker molecule prior to the step of preparative electrophoresis. Subsequent purification of the Kan<sup>r</sup> DNA fragment removed the unligated linker molecules. The purified 1.3 kb Kan<sup>r</sup> fragment was then employed in *in vitro* mutagenesis experiments. In these experiments, partial restriction endonuclease digestions were performed on purified plasmid DNA by limiting the amount of restriction enzyme added to the reaction. By adding the appropriate amount of a given enzyme to a reaction, a high proportion of singly-cut linear molecules was obtained. The appropriate amount of each particular enzyme was determined empirically. Subsequently, the purified Kan<sup>r</sup> fragment is ligated to the partially digested plasmid DNA. Products of this ligation reaction are used to transform *E. coli*, and selection for kanamycin-resistant transformants selects for recombinant plasmid molecules which contain the Kan<sup>r</sup> DNA fragment inserted at some restriction site in the plasmid. Plasmid DNAs from Kan<sup>r</sup> transformants were analyzed to identify the location of particular insertion mutations. Deletion mutations were obtained when the Kan<sup>r</sup> DNA fragment was ligated to a plasmid molecule which had been cut two or more times by the restriction endonuclease. The insertion and deletion mutations constructed in plasmids H336 and HA3 are shown in Figure 15.

In order to analyze the phenotypes of both *in vivo*-and *in vitro*- generated insertion mutations, the mutant plasmids were transferred via conjugation into *X. campestris* Gum<sup>-</sup> deletion mutants. Mutant derivatives of pRK290-H336 were transferred into the deletion strain X1231 where the Gum phenotype will reflect the affect of the insertion mutation carried by the plasmid. The phenotypes of many of the mutations have been analyzed *in vivo* and/or *in vitro* by methods described in Example 2. Plasmids carrying certain insertion mutations could not be transferred into deletion strain X1231. It is most probable that these insertion mutations are lethal or severely deleterious in *X. campestris*. These mutations and other lethal mutations are described in Example 20.

#### Example 20

This example describes the evidence for lethal mutations within the gum gene cluster and discusses the possible functions of the proteins inactivated by these lethal mutations.

As described in Example 19, mutations were isolated in gum gene DNA by *in vitro* insertion of the 1.3 kb Kan<sup>r</sup> fragment into the cloned gum gene DNA carried on pRK290-H336. These insertion mutant plasmids were constructed and analyzed in *E. coli*. In order to assess the Gum phenotypes of these mutants, we subsequently attempted to conjugally transfer mutant plasmids into the Gum<sup>-</sup> deletion strain X1231. Most

mutant plasmids were efficiently transferred into X1231 via standard triparental matings. However, a few mutant plasmids were not transferred into X1231 or transferred at low frequency.

One such mutant plasmid was pRK290-H336.KR9 (KR9). This plasmid contains an insertion into the EcoRI site at position 6089 within the DNA sequence of the gum gene cluster and interrupts the open reading frame encoding gpE as described in Example 17. This is the only insertion isolated to date that interrupts this gene. Our initial attempt to transfer this insertion mutant plasmid, pRK290-H336.KR9, into X1231 failed, as did a repetition of this experiment. This result suggested the possibility that the KR9 insertion was a lethal mutation in X. campestris. We subsequently attempted to transfer KR9 into other X. campestris strains. A mating was performed with recipients X1231, X77 (wild type), and X1205, a Gum<sup>-</sup> deletion strain that lacks the gum DNA between the right-hand BamHI site of the 2.2 kb fragment and the HindIII site of the 11.5 kb BamHI fragment (Figure 15). This strain has an intact copy of the 4.7 kb BamHI fragment and thus an intact copy of the gene inactivated by insertion KR9. In this experiment, KR9 was again not transferred into X1231. However, the plasmid was readily transferred into X77 and X1205. In the X1205 strain, the plasmid resulted in a "gummy" phenotype. This indicates that the gene functions missing from the chromosomal deletion of X1205 were supplied by the corresponding segment of cloned gum gene DNA on the plasmid. These results are consistent with the notion that the KR9 insertion is a lethal mutation. However, the gene inactivated by the KR9 mutation cannot be an essential gene, per se, because this gene is eliminated in many of the large deletion strains such as X1231, X1107, and X1106, which are viable. Thus, it seems that the KR9 mutation is lethal only when the rest of the gum biosynthetic pathway remains operative.

Similar results were observed for two insertion mutant plasmids that carried insertion mutations into the SpeI site at position 11,716 within the sequence. These mutant plasmids, pRK290-H336.KSp12 (KSp12) and pRK290-H336.KSp13 (KSp13), differ only in the orientation of the inserted Kan<sup>r</sup> DNA fragment. In standard triparental matings with a series of X. campestris recipients, both plasmids were efficiently transferred into the Gum<sup>+</sup> recipient X1229 and into the Gum<sup>-</sup> recipient X1217 which carries a wild-type copy of the 3.5 kb BamHI fragment in its chromosome and thus a wild-type copy of the gene encoding gpJ. Transfer of these two plasmids into the Gum<sup>-</sup> recipient X1205 was roughly three orders of magnitude lower, and transfer into the Gum<sup>-</sup> strain X1231 was lower still. The deletion in X1205 removes the 3.5 kb BamHI fragment but is otherwise identical to X1217. Strain X1231 is the largest gum gene deletion and eliminates all of the cloned gum gene DNA carried by pRK290-H336 and its insertion derivatives such as KSp12 and KSp13. These results indicate that the KSp12 and KSp13 insertions may be deleterious or lethal in X. campestris.

Using a somewhat different experimental approach, we have fortuitously discovered that a deletion of the 1.5 kb BamHI fragment appears to be lethal when the remainder of the gum gene cluster is intact. The plasmid pRK290-HA3 (HA3) as shown in Figure 10 contains all the gum gene cluster DNA except the 1.4 kb and 1.5 kb BamHI fragments. We were interested in determining the phenotype of the deletion strain X1106 carrying the HA3 plasmid in order to determine the effect of breaking the gum cluster at the BamHI site delineating the 1.5 and 4.7 kb BamHI segments. Therefore, a derivative of HA3 (HA3.1) carrying a TnK12 insertion within the vector portion of HA3 was transferred into deletion X1106. This strain was found to be mucoid, which indicated that no gene essential for gum biosynthesis spans this BamHI site. Moreover, this shows that the cloned gum genes of HA3.1 are expressed off the plasmid.

However, a more interesting observation was made when we attempted to transfer HA3.1 into deletions X1107 and X1231. We hoped to analyze the phenotypes that would result from effective deletion of the 1.5 kb BamHI segment (in X1107) and deletion of both 1.5 and 1.4 kb BamHI fragments (in X1231). What we found was that HA3.1 could not be transferred into either deletion strain. This experiment was repeated and the result confirmed. HA3.1 was readily transferred into X1106 and X77 (wild type) but could not be transferred in X1107 or X1231. Thus, we conclude that the deletion of the 1.5 kb BamHI segment and deletion of the 1.5 kb and 1.4 kb BamHI segments are both lethal mutations. However, since the deletion strains X1107 and X1231 both lack the 1.5 kb BamHI fragment and are viable, it must be true that the genetic information eliminated by this deletion is not essential, per se. Again, this suggests that lack of the 1.5 kb BamHI segment becomes lethal when the rest of the gum gene pathway is operative.

We have obtained evidence that at least three different mutations in the gum gene cluster have lethal phenotypes. This lethality appears to be manifest only when at least some portion of the gum biosynthetic pathway remains active. Accumulation of a toxic product normally metabolized by the missing function(s) could account for this lethality. Under such a model, the activity of the gum biosynthetic pathway (or some portion of it) would result in lethality if certain other gene functions were absent. For example, such genes might encode the polymerase. Synthesis of C<sub>55</sub> lipid-linked pentasaccharide might be lethal because the C<sub>55</sub> lipid is absolutely required in at least one other cellular function that is essential for growth--cell wall biosynthesis. Thus, sequestering of the C<sub>55</sub> lipid into a non-metabolizable form might cause lethality.

Alternatively, such genes might encode proteins involved in transport of the polymer out of the cell. Synthesis of the polymer in the absence of a transport system might also have deleterious effects on cell growth and could well be lethal. It is also possible that a "lethal" gene might encode Transferase V. No Transferase V mutants have been identified to date. Possibly the Transferase V defect could be lethal because it results in biosynthesis of a polymer (polytetramer) which is toxic to the microbe. For example, polytetramer might not be properly transported by the transport system that normally secretes xanthan.

The hypothesis that blocking gum biosynthesis at an early step in the pathway suppresses lethality could be tested. The experiment would be to construct double mutants between large Gum<sup>-</sup> deletions and sugar nucleotide mutants. A strain defective in UPD-glucose synthesis cannot initiate xanthan biosynthesis and therefore ought not to be subject to this lethality. Therefore, plasmids carrying the lethal insertion mutations ought to be readily transferred into and maintained by such double mutants. If this proved to be true, one could identify the functions encoded by the "lethal genes" through *in vitro* analysis of xanthan biosynthesis where the sugar nucleotides are supplied exogenously. It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

### Claims

Claims for the following Contracting States : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. A recombinant-DNA mediated method for the production of xanthan polysaccharides or xanthan variant polysaccharides structurally related to xanthan comprising:
  - a) preparation of a DNA sequence as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in *E. coli* LE392) or variants thereof capable of directing an non-Xanthomonas host to produce a xanthan polysaccharide or a xanthan variant polysaccharide structurally related to xanthan;
  - b) cloning the DNA sequence into at least one vector capable of being transferred into and replicating in a host microorganism, such vector containing elements for the expression of the biosynthetic enzymes encoded by the DNA sequence;
  - c) transferring the vector containing the DNA sequence into a host microorganism capable of producing polysaccharide under the direction of the DNA sequence;
  - d) culturing the host microorganism under conditions appropriate for maintenance of the vector and synthesis of the polysaccharide; and
  - e) harvesting the polysaccharide.
2. The method of claim 1 wherein the polysaccharide to be produced is xanthan gum.
3. The method of claim 1 wherein the polysaccharide to be produced is polytrimer.
4. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated polytrimer.
5. The method of claim 1 wherein the polysaccharide to be produced is polytetramer.
6. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated polytetramer.
7. The method of claim 1 wherein the polysaccharide to be produced is non-pyruvylated xanthan gum.
8. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated xanthan gum.
9. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated and non-pyruvylated xanthan gum.
10. The method of claim 1 wherein each of the at least one vectors contain one or more DNA sequences encoding enzymes selected from the group consisting of Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase and Polymerase.
11. The method of claim 1 wherein the at least one vector comprises a vector selected from the group consisting of pRK290-H336 contained in *E. coli* LE392 and deposited under ATCC 67049, and pX209



Alternatively, such genes might encode proteins involved in transport of the polymer out of the cell. Synthesis of the polymer in the absence of a transport system might also have deleterious effects on cell growth and could well be lethal. It is also possible that a "lethal" gene might encode Transferase V. No Transferase V mutants have been identified to date. Possibly the Transferase V defect could be lethal  
 5 because it results in biosynthesis of a polymer (polytetramer) which is toxic to the microbe. For example, polytetramer might not be properly transported by the transport system that normally secretes xanthan.

The hypothesis that blocking gum biosynthesis at an early step in the pathway suppresses lethality could be tested. The experiment would be to construct double mutants between large Gum<sup>-</sup> deletions and sugar nucleotide mutants. A strain defective in UPD-glucose synthesis cannot initiate xanthan biosynthesis  
 10 and therefore ought not to be subject to this lethality. Therefore, plasmids carrying the lethal insertion mutations ought to be readily transferred into and maintained by such double mutants. If this proved to be true, one could identify the functions encoded by the "lethal genes" through *in vitro* analysis of xanthan biosynthesis where the sugar nucleotides are supplied exogenously. It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present  
 15 invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

### Claims

Claims for the following Contracting States : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

20

1. A recombinant-DNA mediated method for the production of xanthan polysaccharides or xanthan variant polysaccharides structurally related to xanthan comprising:

- 25 a) preparation of a DNA sequence as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in *E. coli* LE392) or variants thereof capable of directing an non-Xanthomonas host to produce a xanthan polysaccharide or a xanthan variant polysaccharide structurally related to xanthan;
- b) cloning the DNA sequence into at least one vector capable of being transferred into and replicating in a host microorganism, such vector containing elements for the expression of the biosynthetic enzymes encoded by the DNA sequence;
- 30 c) transferring the vector containing the DNA sequence into a host microorganism capable of producing polysaccharide under the direction of the DNA sequence;
- d) culturing the host microorganism under conditions appropriate for maintenance of the vector and synthesis of the polysaccharide; and
- e) harvesting the polysaccharide.

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2. The method of claim 1 wherein the polysaccharide to be produced is xanthan gum.

3. The method of claim 1 wherein the polysaccharide to be produced is polytrimer.

40 4. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated polytrimer.

5. The method of claim 1 wherein the polysaccharide to be produced is polytetramer.

6. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated polytetramer.

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7. The method of claim 1 wherein the polysaccharide to be produced is non-pyruvylated xanthan gum.

8. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated xanthan gum.

50 9. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated and non-pyruvylated xanthan gum.

10. The method of claim 1 wherein each of the at least one vectors contain one or more DNA sequences encoding enzymes selected from the group consisting of Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase and Polymerase.  
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11. The method of claim 1 wherein the at least one vector comprises a vector selected from the group consisting of pRK290-H336 contained in *E. coli* LE392 and deposited under ATCC 67049, and pX209



contained in E. coli LE392 and deposited under ATCC 67051.

12. The method of claim 10 wherein the at least one vector further comprises at least one of the portable DNA sequences encoding the enzymes directing the synthesis of sugar nucleotides, said sugar nucleotides selected from the group consisting of UDP-glucose, UDP-glucuronic acid and GDP-mannose.
13. The method of claim 1 wherein the host is capable of producing said polysaccharide at high synthetic rates.
14. The method of claim 1 wherein the host is capable of producing said polysaccharide at an elevated temperature.
15. The method of claim 14 wherein the host is capable of producing said polysaccharide at a temperature greater than 30 °C.
16. The method of claim 1 wherein the host is capable of producing said polysaccharide under anaerobic conditions.
17. The method of claim 1 wherein said host is a denitrifying bacterium.
18. The method of claim 16 wherein said host is selected from the bacteria of the genus Clostridium.
19. The method of claim 1 wherein said host is selected from the group consisting of Pseudomonas putida, Pseudomonas cepacia, Pseudomonas denitrificans, Pseudomonas fluorescens, Pseudomonas stutzeri, Escherichia coli, and Enterobacter cloacae.
20. The method of claim 1 wherein said variants of said DNA sequence hybridize to DNA from bacteria of the genus Xanthomonas.
21. An article of manufacture comprising a DNA sequence as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in E. coli LE392) or variants thereof capable of directing a microbial cell to synthesize a xanthan polymerase and at least one of the enzymes required for xanthan biosynthesis selected from the group consisting of Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase.
22. The article of manufacture of the claim 21 wherein said variants of said DNA sequence hybridize to DNA from bacteria of the genus Xanthomonas.
23. The plasmid pX209 contained in E. coli LE392 and deposited under ATCC 67051.
24. The plasmid pRK290-H336 contained in E. coli LE392 and deposited under ATCC 67049.
25. A microorganism of the strain E. coli LE392 (pRK290-H336) deposited under ATCC 67049.
26. A microorganism of the strain E. coli LE392 (pX209) deposited under ATCC 67051.

#### Claims for the following Contracting State : AT

1. A recombinant-DNA mediated method for the production of xanthan polysaccharides or xanthan variant polysaccharides structurally related to xanthan comprising:
  - a) preparation of a DNA sequence as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in E. coli LE392) or variants thereof capable of directing an non-Xanthomonas host to produce a xanthan polysaccharide or a xanthan variant polysaccharide structurally related to xanthan;
  - b) cloning the DNA sequence into at least one vector capable of being transferred into and replicating in a host microorganism, such vector containing elements for the expression of the biosynthetic enzymes encoded by the DNA sequence;



- c) transferring the vector containing the DNA sequence into a host microorganism capable of producing polysaccharide under the direction of the DNA sequence;  
d) culturing the host microorganism under conditions appropriate for maintenance of the vector and synthesis of the polysaccharide; and  
5 e) harvesting the polysaccharide.
2. The method of claim 1 wherein the polysaccharide to be produced is xanthan gum.
3. The method of claim 1 wherein the polysaccharide to be produced is polytrimer.
- 10 4. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated polytrimer.
5. The method of claim 1 wherein the polysaccharide to be produced is polytetramer.
- 15 6. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated polytetramer.
7. The method of claim 1 wherein the polysaccharide to be produced is non-pyruvylated xanthan gum.
8. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated xanthan gum.
- 20 9. The method of claim 1 wherein the polysaccharide to be produced is non-acetylated and non-pyruvylated xanthan gum.
10. The method of claim 1 wherein each of the at least one vectors contain one or more DNA sequences encoding enzymes selected from the group consisting of Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase and Polymerase.
- 25 11. The method of claim 1 wherein the at least one vector comprises a vector selected from the group consisting of pRK290-H336 contained in E. coli LE392 and deposited under ATCC 67049, and pX209 contained in E. coli LE392 and deposited under ATCC 67051.
- 30 12. The method of claim 10 wherein the at least one vector further comprises at least one of the portable DNA sequences encoding the enzymes directing the synthesis of sugar nucleotides, said sugar nucleotides selected from the group consisting of UDP-glucose, UDP-glucuronic acid and GDP-mannose.
- 35 13. The method of claim 1 wherein the host is capable of producing said polysaccharide at high synthetic rates.
- 40 14. The method of claim 1 wherein the host is capable of producing said polysaccharide at an elevated temperature.
15. The method of claim 14 wherein the host is capable of producing said polysaccharide at a temperature greater than 30 °C.
- 45 16. The method of claim 1 wherein the host is capable of producing said polysaccharide under anaerobic conditions.
17. The method of claim 1 wherein said host is a denitrifying bacterium.
- 50 18. The method of claim 16 wherein said host is selected from the bacteria of the genus Clostridium.
19. The method of claim 1 wherein said host is selected from the group consisting of Pseudomonas putida, Pseudomonas cepacia, Pseudomonas denitrificans, Pseudomonas fluorescens, Pseudomonas stutzeri, Escherichia coli, and Enterobacter cloacae.
- 55 20. The method of claim 1 wherein said variants of said DNA sequence hybridize to DNA from bacteria of the genus Xanthomonas.



21. A method for the preparation of DNA sequence capable of directing a microbial cell to synthesize a xanthan polymerase and at least one of the enzymes required for xanthan biosynthesis selected from the group consisting of Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase wherein said DNA sequence comprises a DNA insert as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in E. coli LE392) or variants thereof comprising preparing said sequence in a manner known per se.
22. The method of claim 21 wherein said variants of said DNA sequence hybridize to DNA from bacteria of the genus Xanthomonas.
23. A method for the preparation of the plasmid pX 209 comprising isolating said plasmid from E. coli LE 392 deposited under ATCC 67051.
24. A method for the preparation of the plasmid pRK290-H336 comprising isolating said plasmid from E. coli LE 392 deposited under ATCC 67049.
25. A method for the preparation of a microorganism of the strain E. coli LE 392 (pRK290-H336) deposited under ATCC 67049 comprising cultivating said microorganism in a manner known per se.
26. A method for the preparation of a microorganism of the strain E. coli LE 392 (pX 209) deposited under ATCC 67051 comprising cultivating said microorganism in a manner known per se.
27. An article of manufacture comprising a DNA sequence as contained in the plasmid pRK290-H336 deposited under ATCC 67049 (in E. coli LE392) or variants thereof capable of directing a microbial cell to synthesize a xanthan polymerase and at least one of the enzymes required for xanthan biosynthesis selected from the group consisting of Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase.
28. The article of manufacture of the claim 27 wherein said variants of said DNA sequence hybridize to DNA from bacteria of the genus Xanthomonas.
29. The plasmid pX209 contained in E. coli LE392 and deposited under ATCC 67051.
30. The plasmid pRK290-H336 contained in E. coli LE392 and deposited under ATCC 67049.
31. A microorganism of the strain E. coli LE392 (pRK290-H336) deposited under ATCC 67049.
32. A microorganism of the strain E. coli LE392 (pX209) deposited under ATCC 67051.

#### 40 Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

1. Ein Verfahren mittels rekombinanter DNA für die Herstellung von Xanthanpolysacchariden oder Xanthanpolysaccharidvarianten, die mit Xanthan strukturell verwandt sind, umfassend:
  - (a) Herstellen einer DNA-Sequenz, wie sie in dem Plasmid pRK290-H336, hinterlegt unter ATCC 67049 (in E. coli LE392), enthalten ist, oder von Varianten davon, die einen Nicht-Xanthomonaswirt veranlassen können, ein Xanthanpolysaccharid oder eine Xanthanpolysaccharidvariante, die mit Xanthan strukturell verwandt ist, herzustellen;
  - (b) Klonieren der DNA-Sequenz in mindestens einen Vektor, der in einen Wirtsmikroorganismus übertragen und darin repliziert werden kann, wobei dieser Vektor Elemente für die Expression der biosynthetischen Enzyme, die durch die DNA-Sequenz kodiert werden, enthält;
  - (c) Übertragen des Vektors, enthaltend die DNA-Sequenz, in einen Wirtsmikroorganismus, der Polysaccharid unter dem Einfluß der DNA-Sequenz erzeugen kann;
  - (d) Kultivieren des Wirtsmikroorganismus unter geeigneten Bedingungen zum Beibehalten des Vektors und der Synthese des Polysaccharids; und
  - (e) Ernten des Polysaccharids.
2. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid Xanthangummi ist.



3. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid Polytrimer ist.
4. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetyliertes Polytrimer ist.
- 5 5. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid Polytetramer ist.
6. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetyliertes Polytetramer ist.
7. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-pyrovylierter Xanthangummi ist.
- 10 8. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetylierter Xanthangummi ist.
- 15 9. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetylierter und nicht-pyrovylierter Xanthangummi ist.
- 10 10. Verfahren nach Anspruch 1, worin jeder der Vektoren eine oder mehrere DNA-Sequenzen enthält, die Enzyme kodieren, ausgewählt aus der Gruppe, bestehend aus Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase und Polymerase.
- 25 11. Verfahren nach Anspruch 1, worin der Vektor einen Vektor umfaßt, ausgewählt aus der Gruppe, bestehend aus pRK290-H336, enthalten in E. coli LE392 und hinterlegt unter ATCC 67049, und pX209, enthalten in E. coli LE392 und hinterlegt unter ATCC 67051.
- 30 12. Verfahren nach Anspruch 10, worin der Vektor weiterhin umfaßt mindestens eine der übertragbaren DNA-Sequenzen, die die Enzyme kodieren, die die Synthese von Zuckernukleotiden steuern, wobei die Zuckernukleotide ausgewählt werden aus der Gruppe, bestehend aus UDP-Glucose, UDP-Glucuronsäure und GDP-Mannose.
- 35 13. Verfahren nach Anspruch 1, worin der Wirt das Polysaccharid mit hohen Syntheseraten herstellen kann.
14. Verfahren nach Anspruch 1, worin der Wirt das Polysaccharid bei einer erhöhten Temperatur herstellen kann.
- 40 15. Verfahren nach Anspruch 14, worin der Wirt das Polysaccharid bei einer Temperatur von größer als 30 °C herstellen kann.
16. Verfahren nach Anspruch 1, worin der Wirt das Polysaccharid unter anaeroben Bedingungen herstellen kann.
- 45 17. Verfahren nach Anspruch 1, worin der Wirt ein denitrifizierendes Bakterium ist.
18. Verfahren nach Anspruch 16, worin der Wirt ausgewählt wird aus den Bakterien der Gattung Clostridium.
- 50 19. Verfahren nach Anspruch 1, worin der Wirt ausgewählt wird aus der Gruppe, bestehend aus Pseudomonas putida, Pseudomonas cepacia, Pseudomonas denitrificans, Pseudomonas fluorescens, Pseudomonas stutzeri, Escherichia coli und Enterobacter cloacae.
20. Verfahren nach Anspruch 1, worin die Varianten der DNA-Sequenz an DNA der Bakterien der Gattung Xanthomonas hybridisieren.
- 55 21. Ein Produkt, umfassend eine DNA-Sequenz, wie in dem Plasmid pRK290-H336, hinterlegt unter ATCC 67049 (in E. coli LE392), enthalten, oder Varianten davon, die eine mikrobielle Zelle veranlassen können, eine Xanthanpolymerase und mindestens eines der Enzyme zu synthetisieren, die für die Xanthanbiosynthese erforderlich sind, ausgewählt aus der Gruppe, bestehend aus Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase.



22. Produkt nach Anspruch 21, worin die Varianten der DNA-Sequenz an DNA von Bakterien der Gattung Xanthomonas hybridisieren.
23. Das Plasmid pX209, enthalten in E. coli LE392 und hinterlegt unter ATCC 67051.
24. Das Plasmid pRK290-H336, enthalten in E. coli LE392 und hinterlegt unter ATCC 67049.
25. Ein Mikroorganismus des Stamms E. coli LE392 (pRK290-H336), hinterlegt unter ATCC 67049.
26. Ein Mikroorganismus des Stamms E. coli LE392 (pX209), hinterlegt unter ATCC 67051.

#### Patentansprüche für folgenden Vertragsstaat : AT

1. Ein Verfahren mittels rekombinanter DNA für die Herstellung von Xanthanpolysacchariden oder Xanthanpolysaccharidvarianten, die mit Xanthan strukturell verwandt sind, umfassend:
  - (a) Herstellen einer DNA-Sequenz, wie sie in dem Plasmid pRK290-H336, hinterlegt unter ATCC 67049 (in E. coli LE392), enthalten ist, oder von Varianten davon, die einen Nicht-Xanthomonaswirt veranlassen können, ein Xanthanpolysaccharid oder eine Xanthanpolysaccharidvariante, die mit Xanthan strukturell verwandt ist, herzustellen;
  - (b) Klonieren der DNA-Sequenz in mindestens einen Vektor, der in einen Wirtsmikroorganismus übertragen und darin repliziert werden kann, wobei dieser Vektor Elemente für die Expression der biosynthetischen Enzyme, die durch die DNA-Sequenz kodiert werden, enthält;
  - (c) Übertragen des Vektors, enthaltend die DNA-Sequenz, in einen Wirtsmikroorganismus, der Polysaccharid unter dem Einfluß der DNA-Sequenz erzeugen kann;
  - (d) Kultivieren des Wirtsmikroorganismus unter geeigneten Bedingungen zum Beibehalten des Vektors und der Synthese des Polysaccharids; und
  - (e) Ernten des Polysaccharids.
2. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid Xanthangummi ist.
3. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid Polytrimer ist.
4. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetyliertes Polytrimer ist.
5. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid Polytetramer ist.
6. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetyliertes Polytetramer ist.
7. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-pyrovoylierter Xanthangummi ist.
8. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetylierter Xanthangummi ist.
9. Verfahren nach Anspruch 1, worin das herzustellende Polysaccharid nicht-acetylierter und nicht-pyrovoylierter Xanthangummi ist.
10. Verfahren nach Anspruch 1, worin jeder der Vektoren eine oder mehrere DNA-Sequenzen enthält, die Enzyme kodieren, ausgewählt aus der Gruppe, bestehend aus Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase und Polymerase.
11. Verfahren nach Anspruch 1, worin der Vektor einen Vektor umfaßt, ausgewählt aus der Gruppe, bestehend aus pRK290-H336, enthalten in E. coli LE392 und hinterlegt unter ATCC 67049, und pX209, enthalten in E. coli LE392 und hinterlegt unter ATCC 67051.
12. Verfahren nach Anspruch 10, worin der Vektor weiterhin umfaßt mindestens eine der übertragbaren DNA-Sequenzen, die die Enzyme kodieren, die die Synthese von Zuckernukleotiden steuern, wobei die Zuckernukleotide ausgewählt werden aus der Gruppe, bestehend aus UDP-Glucose, UDP-Glucuronsäu-



re und GDP-Mannose.

13. Verfahren nach Anspruch 1, worin der Wirt das Polysaccharid mit hohen Syntheseraten herstellen kann.
- 5 14. Verfahren nach Anspruch 1, worin der Wirt das Polysaccharid bei einer erhöhten Temperatur herstellen kann.
15. Verfahren nach Anspruch 14, worin der Wirt das Polysaccharid bei einer Temperatur von größer als 30 °C herstellen kann.
- 10 16. Verfahren nach Anspruch 1, worin der Wirt das Polysaccharid unter anaeroben Bedingungen herstellen kann.
17. Verfahren nach Anspruch 1, worin der Wirt ein denitrifizierendes Bakterium ist.
- 15 18. Verfahren nach Anspruch 16, worin der Wirt ausgewählt wird aus den Bakterien der Gattung Clostridium.
- 20 19. Verfahren nach Anspruch 1, worin der Wirt ausgewählt wird aus der Gruppe, bestehend aus Pseudomonas putida, Pseudomonas cepacia, Pseudomonas denitrificans, Pseudomonas fluorescens, Pseudomonas stutzeri, Escherichia coli und Enterobacter cloacae.
- 25 20. Verfahren nach Anspruch 1, worin die Varianten der DNA-Sequenz an DNA der Bakterien der Gattung Xanthomonas hybridisieren.
- 30 21. Verfahren zum Herstellen einer DNA-Sequenz, die eine mikrobielle Zelle veranlassen kann, eine Xanthanpolymerase und mindestens eines der Enzyme zu synthetisieren, die für die Xanthanbiosynthese erforderlich sind, ausgewählt aus der Gruppe, bestehend aus Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase, worin die DNA-Sequenz ein DNA-Insert umfaßt wie enthalten in dem Plasmid pRK290-H336, hinterlegt unter ATCC 67049 (in E. coli LE392), oder Varianten davon, umfassend Herstellen der Sequenz in an sich bekannter Weise.
- 35 22. Verfahren nach Anspruch 21, worin die Varianten der DNA-Sequenz an DNA von Bakterien der Gattung Xanthomonas hybridisieren.
- 40 23. Verfahren zur Herstellung des Plasmids pX209, umfassend Isolieren des Plasmids aus E. coli LE392, hinterlegt unter ATCC 67051.
24. Verfahren zur Herstellung des Plasmids pRK290-H336, umfassend Isolieren des Plasmids aus E. coli LE392, hinterlegt unter ATCC 67049.
- 45 25. Verfahren zur Herstellung eines Mikroorganismus des Stamms E. coli LE392 (pRK290-H336), hinterlegt unter ATCC 67049, umfassend Kultivieren des Mikroorganismus in an sich bekannter Weise.
26. Verfahren zur Herstellung eines Mikroorganismus des Stamms E. coli LE392 (pX209), hinterlegt unter ATCC 67051, umfassend Kultivieren des Mikroorganismus in an sich bekannter Weise.
- 50 27. Ein Produkt, umfassend eine DNA-Sequenz, wie in dem Plasmid pRK290-H336, hinterlegt unter ATCC 67049 (in E. coli LE392), enthalten, oder Varianten davon, die eine mikrobielle Zelle veranlassen können, eine Xanthanpolymerase und mindestens eines der Enzyme zu synthetisieren, die für die Xanthanbiosynthese erforderlich sind, ausgewählt aus der Gruppe, bestehend aus Transferase I, Transferase II, Transferase III, Transferase IV, Transferase V, Acetylase, Ketalase.
- 55 28. Produkt nach Anspruch 27, worin die Varianten der DNA-Sequenz an DNA von Bakterien der Gattung Xanthomonas hybridisieren.
29. Das Plasmid pX209, enthalten in E. coli LE392 und hinterlegt unter ATCC 67051.



30. Das Plasmid pRK290-H336, enthalten in E. coli LE392 und hinterlegt unter ATCC 67049.
31. Ein Mikroorganismus des Stamms E. coli LE392 (pRK290-H336), hinterlegt unter ATCC 67049.
- 5 32. Ein Mikroorganismus des Stamms E. coli LE392 (pX209), hinterlegt unter ATCC 67051.

#### Revendications

Revendications pour les Etats contractants suivants : BE, CH, LI, DE, FR, GB, IT, LU, NL, SE

- 10 1. Un procédé par l'intermédiaire d'un ADN recombinant pour la production de polysaccharides de type xanthane ou de polysaccharides de variantes du xanthane, apparenté structurellement au xanthane comportant:
- 15 a) la préparation d'une séquence d'ADN telle que renfermée dans le plasmide pRK290-H336 déposé sous ATCC 67049 (dans E. coli LE392) ou des variantes de celui-ci capable de diriger un hôte non-xanthomonas afin d'obtenir un polysaccharide de type xanthane ou un polysaccharide d'une variante du xanthane structurellement apparenté au xanthane ;
- 20 b) le clonage de la séquence d'ADN en au moins un vecteur apte à être transféré dans et à être répliqué dans un microorganisme hôte, un tel vecteur renfermant des éléments pour l'expression d'enzymes biosynthétiques encodés par la séquence ADN ;
- c) le transfert du vecteur renfermant la séquence d'ADN dans un microorganisme hôte susceptible de produire un polysaccharide sous la direction de la séquence d'ADN ;
- d) la culture du microorganisme hôte sous des conditions appropriées pour l'entretien du vecteur et la synthèse du polysaccharide ; et
- 25 e) la récolte du polysaccharide.
2. Le procédé de la revendication 1, dans lequel le polysaccharide dent être produit est la gomme de xanthane.
3. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytrimère.
- 30 4. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytrimère non-acétylée.
5. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est a polytétramère.
- 35 6. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytétramère non-acétylée.
7. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est une gomme de xanthane non-pyruvylée.
8. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est la gomme de xanthane non-acétylée.
- 45 9. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est la gomme de xanthane non-acétylée et non-pyruvylée.
10. Le procédé de la revendication 1, dans lequel chacun parmi les vecteurs, et au moins l'un d'entre eux renferme une ou plus d'une séquence ADN encodant les enzymes, choisis dans le groupe constitué de la transférase I, la transférase II, la transférase III, la transférase IV, la transférase V, l'acétylase, la cétylase et la polymérase.
- 50 11. Le procédé de la revendication 1, dans lequel au moins un vecteur comporte un vecteur choisi dans le groupe constitué de pRK290-H336 contenu dans E. coli LE392 et déposé sous la dénomination ATCC 67049, et pX209 contenu dans E. coli LE392 et déposé sous ATCC 67051.
- 55



12. Le procédé de la revendication 10, dans lequel au moins un vecteur comporte en outre au moins une des séquences d'ADN portable encodant les enzymes dirigeant la synthèse des nucléotides-sucre, lesdits nucléotides de type sucre étant choisis dans le groupe constitué de IUDP-glucose, de l'acide UDP-glucuronique et du GDP-mannose.
- 5 13. Le procédé de la revendication 1, dans lequel l'hôte est capable de produire le polysaccharide à des vitesses de synthèse élevées.
14. Le procédé de la revendication 1, dans lequel l'hôte est apte à produire ledit polysaccharide à une  
10 température élevée.
15. Le procédé de la revendication 14, dans lequel l'hôte est apte à produire ledit polysaccharide à une température supérieure à 30 °C.
- 16 16. Le procédé de la revendication 1, dans lequel l'hôte est apte à produire ledit polysaccharide sous des conditions anaérobiques.
17. Le procédé de la revendication 1, dans lequel ledit hôte est une bactérie dénitrifiante.
- 20 18. Le procédé de la revendication 1, dans lequel ledit hôte est choisi parmi les bactéries du genre Clostridium.
19. Le procédé de la revendication 1, dans lequel ledit hôte est choisi dans le groupe constitué par Pseudomonas putida, Pseudomonas cepacia, Pseudomonas dinitrificans, Pseudomonas fluorescens,  
25 Pseudomonas stutzeri, Escherichia coli, et Enterobacter cloacae.
20. Le procédé de la revendication 1, dans lequel lesdites variantes de ladite séquence d'ADN s'hybridisent en ADN à partir de bactéries du genre Xanthomonas.
- 30 21. Un article de fabrication comportant une séquence d'ADN telle que contenue dans le plasmide pRK290-H336 déposé sous ATCC 67049 (dans E. coli LE392) ou des variantes de celui-ci aptes à diriger une cellule microbale de façon à synthétiser une polymérase de xanthane et au moins un des enzymes nécessaires pour la biosynthèse du xanthane, choisi dans le groupe constitué de la transférase I, de la transférase II, de la transférase III, de la transférase IV, de la transférase V, de  
35 l'acétylase, de la cétylase.
22. L'article de fabrication de la revendication 21, dans lequel les variantes de ladite séquence d'ADN s'hybridisent en ADN à partir de la bactérie du genre Xanthomonas.
- 40 23. Le plasmide pX209 renfermé dans E. coli LE392 et déposé sous ATCC 67051.
24. Le plasmide pRK290-H336 contenu dans la souche E. coli LE392 et déposé sous ATCC 67049.
25. Un microorganisme de la souche E.coli LE392 (pRK290-H336) déposé sous ATCC 67049.
- 45 26. Un microorganisme de la souche E. coli LE392 (pX209) déposé sous ATCC 67051.

#### Revendications pour l'Etat contractant suivant : AT

- 50 1. Un procédé par l'intermédiaire d'un ADN recombinant pour la production de polysaccharides de type xanthane ou de polysaccharides de variantes du xanthane, apparenté, structurellement au xanthane comportant :
  - a) la préparation d'une séquence d'ADN telle que renfermée dans le plasmide pRK290-H336 déposé sous ATCC 67049 (dans E. coli LE392) ou des variantes de celui-ci capable de diriger un hôte non-xanthomonas afin d'obtenir un polysaccharide du type xanthane ou un polysaccharide d'une variante  
55 du xanthane structurellement apparenté au xanthane ;
  - b) le clonage de la séquence d'ADN en au moins un vecteur apte à être transféré dans et à être répliqué dans un microorganisme hôte, un tel vecteur renfermant des éléments pour l'expression



- d'enzymes biosynthétiques encodés par la séquence ADN ;  
c) le transfert du vecteur renfermant la séquence d'ADN dans un microorganisme hôte susceptible de produire un polysaccharide sous la direction de la séquence d'ADN ;  
d) la culture du microorganisme hôte sous des conditions appropriées pour l'entretien du vecteur et la synthèse du polysaccharide ; et  
e) la récolte du polysaccharide.
2. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est la gomme de xanthane.
3. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytrimère.
4. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytrimère non-acétylée.
5. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytétramère.
6. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est un polytétramère non-acétylée.
7. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est une gomme de xanthane non-pyruvylée.
8. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est la gomme de xanthane non-acétylée.
9. Le procédé de la revendication 1, dans lequel le polysaccharide devant être produit est la gomme de xanthane non-acétylée et non-pyruvylée.
10. Le procédé de la revendication 1, dans lequel chacun parmi les vecteurs, et au moins l'un d'entre eux renferme une ou plus d'une séquence ADN encodant les enzymes, choisis dans le groupe constitué de la transférase I, la transférase II, la transférase III, la transférase IV, la transférase V, l'acétylase, la cétalase et la polymérase.
11. Le procédé de la revendication 1, dans lequel au moins un vecteur comporte un vecteur choisi dans le groupe constitué de pRK290-H336 contenu dans *E. coli* LE392 et déposé sous la dénomination ATCC 67049, et pX209 contenu dans *E. coli* LE392 et déposé sous ATCC 67051.
12. Le procédé de la revendication 10, dans lequel au moins un vecteur comporte en outre au moins une des séquences d'ADN portable encodant les enzymes dirigeant la synthèse des nucléotides-sucres, lesdits nucléotides de type sucre étant choisis dans le groupe constitué de l'UDP-glucose, l'acide UDP-glucuronique et du GDP-mannose.
13. Le procédé de la revendication 1, dans lequel l'hôte est capable de produire le polysaccharide à des vitesses de synthèse élevées.
14. Le procédé de la revendication 1, dans lequel l'hôte est apte à produire ledit polysaccharide à une température élevée.
15. Le procédé de la revendication 14, dans lequel l'hôte est apte à produire ledit polysaccharide à une température supérieure à 30 °C.
16. Le procédé de la revendication 1, dans lequel l'hôte est apte à produire ledit polysaccharide sous des conditions anaérobiques.
17. Le procédé de la revendication 1, dans lequel ledit hôte est une bactérie dénitrifiante.



18. Le procédé de la revendication 1, dans lequel ledit hôte est choisi parmi les bactéries du genre Clostridium.
- 5 19. Le procédé de la revendication 1, dans lequel ledit hôte est choisi dans le groupe constitué par Pseudomonas putida, Pseudomonas cepacia, Pseudomonas dinitrificans, Pseudomonas fluorescens, Pseudomonas stutzeri, Escherichia coli, et Enterobacter cloacae.
20. Le procédé de la revendication 1, dans lequel lesdites variantes de ladite séquence d'ADN s'hybridisent en ADN à partir de bactéries du genre Xanthomonas.
- 10 21. Un procédé pour la préparation d'une séquence d'ADN apte à diriger une cellule microbienne de façon à synthétiser une polymérase de xanthane et au moins un des enzymes nécessaires pour la biosynthèse du xanthane, choisi dans le groupe constitué de la transférase I, de la transférase II, de la transférase III, de la transférase IV, de la transférase V, de l'acétylase, de la cétylase, procédé dans lequel ladite séquence d'ADN comporte un insert d'ADN tel que contenu dans le plasmide pRK290-H336 déposé sous ATCC 67049 (dans E. coli LE392) ou des variantes de celui-ci, procédé selon lequel on prépare ladite séquence d'une manière connue en soi.
- 15 22. Un procédé de la revendication 21, dans lequel les variantes de ladite séquence d'ADN s'hybridisent de l'ADN à partir de la bactérie du genre Xanthomonas.
- 20 23. Un procédé pour la préparation du plasmide pX209, comportant l'isolation dudit plasmide à partir du E. coli LE392 et déposé sous ATCC 67051.
- 25 24. Un procédé pour la préparation du plasmide pRK290-H336, comportant l'isolation dudit plasmide à partir d'E. coli LE392 et déposé sous ATCC 67049.
- 30 25. Un procédé pour la préparation d'un microorganisme de la souche E. coli LE392 (pRK290-H336) déposé sous ATCC 67049, comportant la culture dudit microorganisme d'une manière connue en soi.
- 35 26. Un procédé pour la préparation d'un microorganisme de la souche E. coli LE392 (pX209) déposé sous ATCC 67051, comportant la culture dudit microorganisme d'une manière connue en soi.
- 40 27. Un article de fabrication comportant une séquence d'ADN telle que contenue dans le plasmide pRK290-H336 déposé sous ATCC 67049 (dans E. coli LE392) ou des variantes de celui-ci aptes à diriger une cellule microbienne de façon à synthétiser une polymérase de xanthane et au moins un des enzymes nécessaires pour la biosynthèse du xanthane, choisi dans le groupe constitué de la transférase I, de la transférase II, de la transférase III, de la transférase IV, de la transférase V, de l'acétylase, de la cétylase.
- 45 28. L'article de fabrication de la revendication 21, dans lequel les variantes de ladite séquence d'ADN s'hybridisent en ADN à partir de la bactérie du genre Xanthomonas.
29. Le plasmide pX209 renfermé dans E. coli LE392 et déposé sous ATCC 67051.
30. Le plasmide pRK290-H336 contenu dans E. coli LE392 et déposé sous ATCC 67049.
31. Un microorganisme de la souche E. coli LE392 (pRK290-H336) déposé sous ATCC 67049.
- 50 32. Un microorganisme de la souche E. coli LE392 (pX209) déposé sous ATCC 67051.
- 55