Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2571518 C 2018/01/02

(11)(21) 2 571 518

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2005/06/20
(87) Date publication PCT/PCT Publication Date: 2006/01/26
(45) Date de délivrance/Issue Date: 2018/01/02
(85) Entrée phase nationale/National Entry: 2006/12/20
(86) N° demande PCT/PCT Application No.: US 2005/021637
(87) N° publication PCT/PCT Publication No.: 2006/009938
(30) Priorité/Priority: 2004/06/21 (US60/580,730)

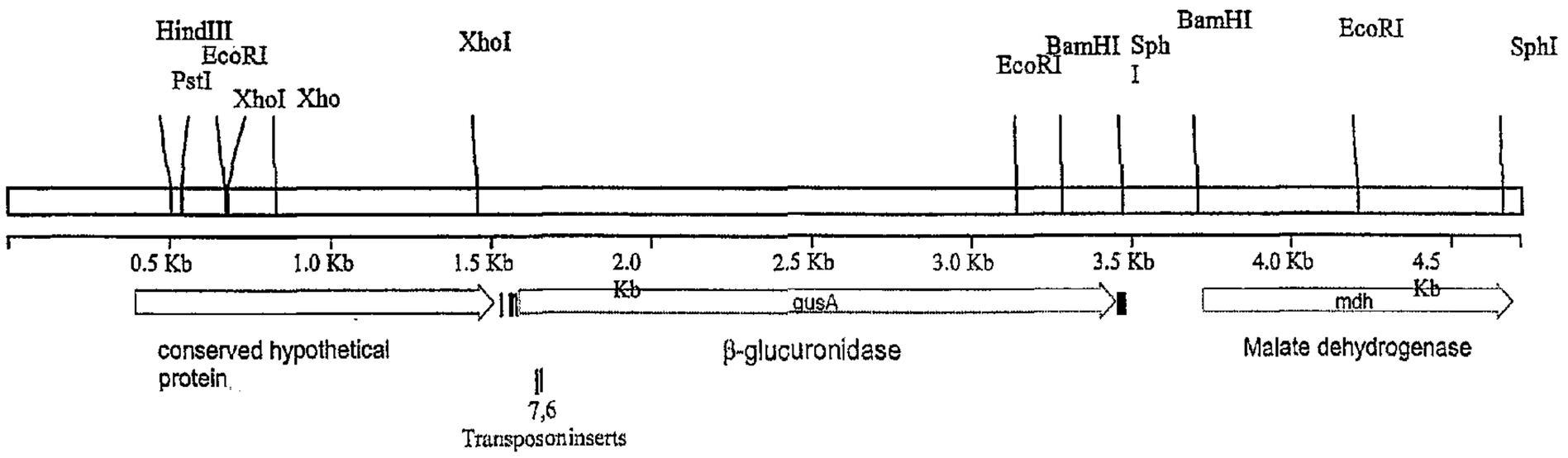
(51) Cl.Int./Int.Cl. *C12N 15/56* (2006.01), *C08L 5/00* (2006.01), *C12N 1/20* (2006.01), *C12N 1/21* (2006.01), *C12N 15/00* (2006.01), *C12N 15/53* (2006.01), *C12N 15/55* (2006.01), *C12N 9/04* (2006.01), *C12N 9/16* (2006.01), *C12N 9/24* (2006.01), *C12P 19/04* (2006.01)

(72) Inventeurs/Inventors:
CLEARY, JOSEPH M., US;
COLEMAN, RUSSELL J., US;
HARDING, NANCY E., US;
PATEL, YAMINI N., US

(73) Propriétaire/Owner:
CP KELCO U.S., INC., US

(74) Agent: MOFFAT & CO.

(54) Titre : GOMME GELLANE GENETIQUEMENT PURIFIEE
(54) Title: GENETICALLY PURIFIED GELLAN GUM



(57) Abrégé/Abstract:
Mutational inactivation of proteins involved in para-cresol production in certain milk products results in improved taste and odor. The undesirable para-cresol forms over time as a result of enzymes produced by the bacterium that produces gellan gum. Since the gellan is typically used in a relatively unpurified form, the enzymes are added to the milk along with the gellan. Inactivation of the enzymes is a genetic means of eliminating the enzymes without requiring any additional purification or processing.

ABSTRACT

Mutational inactivation of proteins involved in para-cresol production in certain milk products results in improved taste and odor. The undesirable para-cresol forms over time as a result of enzymes produced by the bacterium that produces gellan gum. Since the gellan is typically used in a relatively unpurified form, the enzymes are added to the milk along with the gellan. Inactivation of the enzymes is a genetic means of eliminating the enzymes without requiring any additional purification or processing.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2 __

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

GENETICALLY PURIFIED GELLAN GUM

FIELD OF THE INVENTION

[02] The invention relates to the field of food additives. In particular it relates to the field of dairy food additives. More particularly it relates to additives to sterilized milk products.

BACKGROUND OF THE INVENTION

[03] Gellan gum is an extracellular polysaccharide produced by the bacteria *Sphingomonas elodea.* Gellan gum produced by *S. elodea* is commercially available as Kelcogel LT100® from CP Kelco, San Diego, CA. Commercially, gellan gum is formed by aerobic fermentation. Upon completion of fermentation, the broth is pasteurized to kill viable cells prior to recovery of the gum from the fermentation broth.

[04] Gellan gum comprises the sugars glucose, glucuronic acid, and rhamnose in a 2:1:1 molar ratio, which are linked to form a tetrasaccharide repeat unit. Native gellan gum is acetylated and glycerylated on the same glucose residues. On average, there is one acetyl group and one half glyceryl group per tetrasaccharide repeat unit.

[05] The method of recovery of the gellan gum affects the characteristics of the gum. Direct recovery yields a soft, flexible gel. Gellan gum has long been used in cultured, retorted, and frozen dairy products due to its textural and rheological properties.

However, an off-flavor and odor develop in otherwise shelf-stable, milk-based, gellan-containing products; this flavor and odor render the foods unpalatable. The off-flavor and odor have been linked to the formation of para-cresol from substrates in milk, *e.g.,* para-cresyl sulfate and para-cresyl glucouronide. Para-cresol is detectable in milk-based, gellan-containing products that have been treated at ultra high temperatures and stored at room temperature.

[06] In an effort to eliminate this problem, gellan has been deacylated with hot alkali treatment. While effective in eliminating the para-cresol, the deacylation processing makes the gellan gum more brittle and less useful for certain food applications. Another approach to eliminate this problem is the pre-treatment of native gellan gum with a denaturing agent, such as sodium hypochlorite or potassium hydroxide. This approach adds material and processing costs. There is a need in the art for a gellan product which does not produce para-cresol upon prolonged storage in a sterilized dairy product and which does not require extra processing steps.

BRIEF SUMMARY OF THE INVENTION

[07] In a first embodiment a composition is provided which comprises gellan gum substantially free of arylsulfatase protein.

[08] In a second embodiment a composition is provided which comprises gellan gum substantially free of β-glucuronidase protein.

[09] In a third embodiment of the invention a composition is provided which comprises gellan gum substantially free of both arylsulfatase and β-glucuronidase proteins.

[10] In a fourth embodiment of the invention a method is provided for producing a gellan gum composition. *Sphingomonas elodea* is cultured in a culture medium. The *Sphingomonas elodea* produces no catalytically active arylsulfatase, or no

catalytically active β-glucuronidase, or no catalytically active arylsulfatase and no catalytically active β-glucuronidase. The culture medium is collected. Gellan gum is precipitated from the culture medium.

[11] A microbiologically pure culture of *Sphingomonas elodea* is provided in a fifth embodiment of the invention. It is arylsulfatase-deficient.

[12] Another microbiologically pure culture of *Sphingomonas elodea* is provided in a sixth embodiment of the invention. It is β-glucuronidase-deficient.

[13] Still another embodiment of the invention is a microbiologically pure culture of *Sphingomonas elodea*. It is deficient in both arylsulfatase and β-glucuronidase.

[14] An eighth embodiment of the invention provides an isolated and purified polynucleotide encoding a *Sphingomonas elodea* arylsulfatase. The arylsulfatase has an amino acid sequence according to SEQ ID NO: 2.

[15] A ninth embodiment of the invention provides an isolated and purified polynucleotide encoding a *Sphingomonas elodea* β-glucuronidase. The β-glucuronidase has an amino acid sequence according to SEQ ID NO: 5.

[16] A tenth embodiment of the invention is an isolated and purified polynucleotide comprising *Sphingomonas elodea* genomic DNA. The genomic DNA comprises a deletion of all or part of its arylsulfatase coding sequence.

[17] An eleventh embodiment of the invention is an isolated and purified polynucleotide comprising *Sphingomonas elodea* genomic DNA. The genomic DNA comprises a deletion of all or part of its β-glucuronidase coding sequence.

[17a] A further embodiment of the invention provides a gellan gum substantially free of catalytically active arylsulfatase protein and substantially free of any denaturing agent and substantially free of denatured arylsulfatase protein.

[17b] In another embodiment, the invention provides a gellan gum substantially free of catalytically active β-glucuronidase protein and substantially free of any denaturing agent and substantially free of denatured β-glucuronidase protein.

[17c] Another embodiment of the invention provides a gellan gum substantially free of catalytically active arylsulfatase and catalytically active β-glucuronidase proteins and substantially free of any denaturing agent and substantially free of denatured arylsulfatase and β-glucuronidase proteins.

[17d] Yet another embodiment of the invention provides a culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum as described herein, wherein the *Sphingomonas elodea* is arylsulfatase deficient and/or is β-glucuronidase deficient and which comprises a deletion, insertion or inactivating mutation in all or part of an arylsulfatase gene, a β-glucuronidase gene, or in both the arylsulfatase gene and the β-glucuronidase gene.

[17e] In another embodiment, the invention provides a precipitated culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum as described herein, wherein the *Sphingomonas elodea* is arylsulfatase deficient and/or is β-glucuronidase deficient and which comprises a deletion, insertion or inactivating mutation in all or part of an arylsulfatase gene, a β-glucuronidase gene, or in both the arylsulfatase gene and the β-glucuronidase gene.

[17f] A further embodiment of the invention provides a sterilized milk product comprising a gellan gum as described herein.

[17g] In another embodiment, the invention provides a method of producing a gellan gum comprising:

growing *Sphingomonas elodea* in a culture medium, wherein the *Sphingomonas elodea* produces no catalytically active arylsulfatase or no catalytically active β-glucuronidase or no catalytically active arylsulfatase and no catalytically active β-glucuronidase;

collecting the culture medium;

precipitating gellan gum from the culture medium.

In yet another embodiment, the method further comprises adding the gellan gum to a dairy product.

[17h] Another embodiment of the invention provides a microbiologically pure culture of *Sphingomonas elodea* which is arylsulfatase deficient and/or β-glucuronidase deficient and which comprises a deletion, insertion or inactivating mutation in all or part of an arylsulfatase gene, a β-glucuronidase gene, or in both of the arylsulfatase gene and the β-glucuronidase gene.

[17i] Yet another embodiment of the invention provides a gellan gum substantially free of catalytically active arylsulfatase protein or catalytically active β-glucuronidase protein and substantially free of any denaturing agent and substantially free of denatured arylsulfatase protein or β-glucuronidase protein, wherein the gellan gum is made by the microbiologically pure culture as described herein.

[17j] In another embodiment, the invention provides an isolated and purified polynucleotide encoding a *Sphingomonas elodea* arylsulfatase comprising an amino acid sequence according to SEQ ID NO: 2. In a further embodiment, the isolated and purified polynucleotide comprises a nucleotide sequence according to nucleotides 521 to 2176 of SEQ ID NO: 1.

[17k] A further embodiment of the invention provides a method of producing isolated and purified mutated *Sphingomonas elodea* DNA which is useful in production of the gellan gum as described herein, the method comprising:

producing a mutation in an arylsulfatase coding sequence of *Sphingomonas elodea* DNA, wherein the arylsulfatase coding sequence corresponds to SEQ ID NO: 1, wherein the mutation comprises a deletion of one or more of nucleotides 521 to 2176 of the arylsulfatase coding sequence, and wherein the mutation results in reduction or loss of arylsulfatase activity; and

isolating and purifying all or part of the mutated *Sphingomonas elodea* DNA.

In another embodiment, the isolated and purified mutated *Sphingomonas elodea* DNA comprises at least a portion of a sequence adjacent to and upstream of a start codon of an arylsulfatase gene ligated to a portion of a sequence adjacent to and downstream of a stop codon of the arylsulfatase gene.

[17l] Yet another embodiment of the invention provides a method of producing isolated and purified mutated *Sphingomonas elodea* DNA which is useful in production of the gellan gum as described herein, the method comprising:

producing a mutation in a β-glucuronidase coding sequence of *Sphingomonas elodea* DNA, wherein the β-glucuronidase coding sequence corresponds to SEQ ID NO: 4, wherein the mutation comprises a deletion of one or more of nucleotides 1588 to 3447 of the β-glucuronidase coding sequence, and wherein the mutation results in reduction or loss of β-glucuronidase activity; and

isolating and purifying all or part of the mutated *Sphingomonas elodea* DNA.

In another embodiment, the isolated and purified mutated *Sphingomonas elodea* DNA comprises at least a portion of a sequence adjacent to and upstream of a start codon of a β-glucuronidase gene ligated to a portion of a sequence adjacent to and downstream of a stop codon of the β-glucuronidase gene.

[17m] Another embodiment of the invention provides an isolated and purified mutated *Sphingomonas elodea* DNA produced by a method as described herein.

[17n] In a further embodiment , the invention provides an isolated and purified polynucleotide which is useful in production of the gellan gum as described herein, the isolated and purified polynucleotide comprising a nucleotide sequence corresponding to SEQ ID NO: 1 in which one or more of nucleotides 521 to 2176 have been deleted such that the isolated and purified polynucleotide encodes an inactive arylsulfatase or an arylsulfatase of reduced activity.

[17o] Still another embodiment of the invention provides an isolated and purified polynucleotide which is useful in production of the gellan gum as described herein, the isolated and purified polynucleotide comprising a nucleotide sequence corresponding to SEQ ID NO: 4 in which one or more of nucleotides 1588 to 3447 have been deleted such that the isolated and purified polynucleotide encodes an inactive β-glucuronidase or a β-glucuronidase of reduced activity.

[18] These and other embodiments of the invention as described in more detail below provide the art with cost-effective means to make a more consumer-acceptable, sterilized, gellan-containing, dairy product.

BRIEF DESCRIPTION OF THE DRAWINGS

[19] Figure 1. Genetic map of the genomic region around the arylsulfatase gene (*atsA*) and location of the regions amplified by PCR and cloned into plasmid pLO2. Plasmid pLO2 with the cloned PCR fragments was then used to replace this region of the genome with the deletion, by homologous recombination.

[20] Figure 2. Restriction map of the genomic region around the beta-glucuronidase gene (*gusA*) of *Sphingomonas elodea.* Positions of transposon insertions in clones BG-6 and BG-7 are indicated at the bottom.

DETAILED DESCRIPTION OF THE INVENTION

[21] The present inventors have found that if either of the genes encoding the enzymes arylsulfatase and β-glucuronidase or both genes are mutationally inactivated in the bacterium *Sphingomonas elodea*, the bacterium produces a gellan that has superior properties for certain purposes. In particular, the gellan that is produced by such mutants imparts to sterilized milk products a longer shelf-life.

[22] If one or both of the enzymes are not inactivated then they produce para-cresol from substrates (p-cresyl-sulfate and p-cresyl-glucuronide) found in the milk. The para-cresol imparts an odor and flavor that is generally unpalatable to consumers. Eliminating these enzymes reduces the rate at which para-cresol is produced in the sterilized milk or milk product on the shelf.

[23] Mutations in either or both of the enzymes may be used to reduce the rate of para-cresol production. The mutations are preferably of the type that totally inactivates the

protein, such as insertions, nonsense, frameshift, or deletion mutations. Any technique known in the art for producing such mutations may be used. The applicants used a transposon insertion strategy to identify the genes encoding arylsulfatase and β-glucuronidase. A deletion mutation in each gene was then constructed by homologous recombination using 5' and 3' DNA fragments flanking the gene which were joined together. Nonetheless, other strategies can be used to obtain the mutations in these genes. The mutations can be made directly in a gellan "production" strain, or the mutations can be transferred to such a strain from a strain in which the mutation is first made. Techniques for site-directed mutagenesis are well known in the art. *See, e.g.*, "In Vitro Mutagenesis Protocols, second edition, Braman, Jeff, ed., Humana Press, 2002, and the commercially available QuikChange™ kit (Stratagene). Provided with the wild-type sequences of the *S. elodea* arylsulfatase and β-glucuronidase genes, one of skill in the art can readily make a variety of desired mutations in these genes.

[24] The sequence of the wild-type and mutant genes encoding arylsulfatase and β-glucuronidase have been determined. See SEQ ID NO: 1 (wild-type arylsulfatase), SEQ ID NO: 3 (deletion of arylsulfatase), SEQ ID NO: 4 (wild-type β-glucuronidase), and SEQ ID NO: 6 (deletion of β-glucuronidase). The identification of these genes and their nucleotide sequences permits one of skill in the art to readily make other mutations having the desired null phenotype for expression of these enzymes. Mutations such as insertions, deletions, nonsense, and frameshift are most likely to result in a null phenotype for the enzymes. Missense mutations can also be made and routinely tested for their effect on enzyme activity. Standard techniques of microbial genetics can be used to make suitable mutations. See, *e.g.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, R. W. Old, S. B. Primrose, Blackwell Publishing, 1994. Standard enzyme assays can

be used to test for loss of activity of the mutated enzymes. See, *e.g.*, Kim *et al.*, *Appl Microbiol Biotechnol.* 2004 Feb; 63(5):553-9.

[25] Compositions of the present invention which are substantially free of arylsulfatase or β-glucuronidase contain less than 95%, 96%, 97%, 98%, or 99% of the amount of the particular protein than is contained in wild-type strains. Such a reduction in amount of enzyme should lead to sterilized milk compositions which have less than 90 %, 93 %, 95 %, 97 %, or 99 % of the amount of para-cresol that is produced in compositions containing gellan from wild-type strains. The amount of arylsulfatase or β-glucuronidase protein which is produced can be measured by enzyme assay using a readily assayable substrate such as 5-bromo-4-chloro-3-indolyl sulfate (X-SO4); CAS No. 6578-07-0 from Sigma or using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA); CAS No. 114162-64-0, from Sigma or RPI Corp. or using p-nitrocatechol. Alternatively the protein can be measured using an immunological technique such as a Western blot.

[26] Gellan gum is typically used in sterilized or ultra high temperature (UHT) treated dairy products or frozen dairy products. Such products include without limitation, ice cream, frozen yogurt, pudding, whipped dairy product, coffee creamer, crème brulee, and dairy beverages. The gellan gum of the present invention can be used in these or any other foods as can typical gellan from a wild-type strain. Gellan gum is typically used for suspension of fine particles, but it also can be used to impart a favorable mouth feel.

[27] Gellan gum can be produced using the mutant strains of the present invention according to any of the methods known in the art for wild-type strains. The bacteria are typically grown in a liquid culture medium. Such medium typically contains a carbon source, phosphate, organic and inorganic nitrogen sources, and appropriate trace elements. The fermentation is typically conducted under sterile conditions, with

aeration and agitation. At the end of the fermentation period, the culture medium is collected, typically without removing the cells. The fermentation broth can be pasteurized to kill viable cells prior to recovery of the gellan gum. The gellan gum can be precipitated as is known in the art. Typically this is done with an alcohol, such as isopropanol. The precipitated gellan can be dried prior to rehydration.

[28] Para-cresol can be measured by any means known in the art. One method which can be used employs dichloromethane extraction, concentration, and gas chromatographic-mass spectroscopy.

[29] While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the scope of the invention as set forth in the appended claims, which should be given the broadest interpretation consistent with the description as a whole.

EXAMPLES

Example 1

[30] The following reagents were used in screening and characterizing mutant strains:

5-bromo-4-chloro-3-indolyl sulfate (X-SO_4); CAS No. 6578-07-0
Source: Sigma.

5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA); CAS No. 114162-64-0,
Source: Sigma or RPI Corp.

Example 2

[31] The genes encoding arylsulfatase and β-glucuronidase were identified by making a library of random transposon mutants in a nonmucoid strain of *S. elodea*, Gρs2, using

the commercially available EZ::TN™ <R6Kgamma-ori /KAN-2> insertion kit from Epicentre (Madison, WI). Kanamycin resistant mutant strains were screened for lack of (or significantly reduced) blue color on selective media with specific chromogenic substrates. See Example 1. Mutants blocking arylsulfatase production or activity were identified using the chromogen 5-bromo-4-chloro-3-indolyl sulfate (X-SO4) on agar with a defined medium with chloride salts. Mutants of β-glucuronidase were selected on a defined medium with the chromogen 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA).

[32] The transposon and adjacent genomic DNA were subsequently excised from the chromosome using restriction enzymes. The restriction enzyme fragments were circularized with ligase and transformed into *Escherichia coli* where the transposon-containing DNA can replicate due to presence of a replicon in the transposon. Plasmid DNA was purified and sequenced. The plasmid with the gene for arylsulfatase was designated R6K-AS#14E. A portion of this plasmid has been sequenced (SEQ ID NO: 1). The plasmid with the gene for beta-glucuronidase was designated R6K-BG#6S. A portion of this plasmid has been sequenced (SEQ ID NO: 4.) These plasmids in *Escherichia coli* strain EC100D pir+ are being deposited at the American Type Culture Collection, Manassas, VA, on June 21, 2004.

[33] In the bacterium that had an inactivated arylsulfatase, the transposon had actually inserted in a gene for a hypothetical protein that was adjacent to the gene for arylsulfatase. In the bacterium that had an inactivated β-glucuronidase, the transposon insertion was located in the amino portion of the gene for β-glucuronidase. DNA sequencing of the genes showed that they were homologous to known genes from other species in the database of the National Center for Biotechnology Information (NCBI).

[34] The genes for arylsulfatase and β-glucuronidase and adjacent genomic DNA were sequenced. Deletions of the genes were constructed on a plasmid and then transferred into *S. elodea* strains S-60wtc and PDG-1. See WO 01/64897. The deletions were inserted in the genome by homologous recombination. DNA sequences flanking the target gene were amplified by PCR and cloned into the plasmid vector pLO2. (Lenz, O., E. Schwartz, J. Dernedde, M. Eitinger and B. Friedrich. 1994, "The *Alcaligenes eutrophus* H16 hoxX gene participates in hydrogenase regulation," *J. Bacteriol. 176:*4385-4393.) This construct was transferred into the *S. elodea* strain by conjugation. The resulting kanamycin resistant strains were then grown for 30-40 generations in the absence of antibiotic. Isolates that had lost the plasmid were detected by selection for sucrose tolerance due to loss of the *sacB* gene on pLO2, and confirmed by kanamycin sensitivity. Isolates that had lost the plasmid after the non-selective growth were of two types, deletion or wild-type. The desired deletion strains were identified by lack of blue color on appropriate indicator agar (see example 1) and by diagnostic PCR. The scheme for construction is shown in Figure 1 below.

[35] To construct a precise deletion of the gene (*atsA*) for arylsulfatase it was necessary to determine the most likely start codon for the *atsA* gene and the start and stop codons of the adjacent genes. The locations of the ends of the genes were determined based on DNA sequences, homologies to other genes in GenBank and third base GC preference using the FramePlot-3 program. Ishikawa and Hotta. "FramePlot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content." *FEMS Microbiology Letters* 174:251-253 (1999). This analysis indicated that the arylsulfatase gene is translationally coupled to the gene for the conserved hypothetical protein, *i.e.,* the stop codon of the arylsulfatase gene overlaps with the start codon of the gene for the conserved

hypothetical protein. The arylsulfatase deletion was constructed to leave the alkylsulfatase gene and the gene for the unknown protein intact, since it is not known whether these proteins are required for optimal cell growth and gellan production.

[36] PCR primers AS5 (CCGAGCTCAACGCCTTCGACTATGTCCA; SEQ ID NO: 11) and AS6 (CCTCTAGACTGGGGATTGTCCGGAAAAG; SEQ ID NO: 12) were used to amplify a 512 bp fragment just upstream of the start codon of *atsA* as a *Sac*I-*Xba*I fragment (total 528 bp). Primers AS3 (CGTCTAGATCCACCCCGGCGACCTTCCC; SEQ ID NO: 13) and AS4 (TATAGCATGCGGCGACCACGGGCTCCTCCTCA; SEQ ID NO: 14) were used to amplify a 479 bp fragment including the end of the *atsA* gene and the start of the conserved hypothetical protein as an *Xba*I-*Sph*I fragment (total 497 bp). Thus, the stop codon of *atsA* was retained but the start codon was deleted, so no portion of the arylsulfatase protein should be synthesized. Restriction sites for cloning were added to the ends of the primers. The PCR fragments were ligated sequentially into the polylinker of plasmid vector pLO2, to form the deletion of the *atsA* gene. The upstream *Sac*I-*Xba*I fragment was cloned into *Sac*I-*Xba*I cut pLO2. Subsequently the downstream *Xba*I-*Sph*I fragment was cloned (Figure 2). This plasmid with deletion of the *atsA* gene was transferred by conjugation into *S. elodea* strains S60wtc and PDG-1 to allow recombination of the plasmid into the chromosome. Kanamycin resistant isolates were purified, then grown in the absence of antibiotic and plated on medium with sucrose and X-SO_4 to select isolates with sucrose tolerance due to loss of the plasmid-encoded *sacB* gene. Sucrose resistance, kanamycin sensitive, yellow colonies were selected. The *atsA* derivatives of S60wtc and PDG-1 were designated GAS-1 and PAS-1 respectively.

[37] A deletion of the gene (*gusA*) for β-glucuronidase was constructed on plasmid pLO2 and transferred into S60wtc, GAS-1. The most likely start codon for the *gusA* gene was determined by homology to other proteins and the presence of ribosome binding

sites. A region of secondary structure is upstream of the start codon. The deletion of the *gusA* gene was constructed to maintain secondary structures upstream and downstream of *gusA*. Primers Bgluc3 (AACTGCAGACACGTGGCTTGTGCCGAAC; SEQ ID NO: 7) and Bgluc4 (GGCTCTAGACTTCTCCCTGTTCCTCCGGGAAA; SEQ ID NO: 8) were used to amplify a 560 bp fragment upstream of the *gusA* gene as a *PstI-XbaI* fragment (total 577 bp). Primers Bgluc1 (TTTCTAGATGACTGTCCAGGCCCCTCTC; SEQ ID NO: 9) and Bgluc2 (TCGAGCTCCAATGTCCTCGTAGCTGTTC; SEQ ID NO: 10) were used to amplify a 489 bp fragment downstream of *gusA* as an *Xba*I-*Sac*I fragment (total 505 bp). The *Pst*I-*Xba*I fragment was cloned into *Pst*I-*Xba*I cut pLO2. Subsequently the downstream *Xba*I-*Sac*I fragment was cloned. This plasmid with deletion of the *gusA* gene was transferred by conjugation into *S. elodea* strains S60wtc, GAS-1 and PAS-1 to allow recombination. Kanamycin resistant isolates were purified, grown in the absence of antibiotic and then plated on media with sucrose and X-GlcA to select isolates with sucrose tolerance due to loss of the plasmid-encoded *sacB* gene. A mixture of blue-green (wild-type) and light green (mutant) colonies was obtained. Sucrose resistant, light green isolates were confirmed for plasmid loss by kanamycin sensitivity. The *gusA* deletion derivatives of S60wtc, GAS-1 and PAS-1 were designated GBG, GBAD and PBAD respectively.

[38] The deletion of the gene (*atsA*) for arylsulfatase in strains S60wtc and PDG-1 has been completed. The gene for β-glucuronidase has been identified and sequenced. The adjacent DNA was sequenced. A deletion of the gene for β-glucuronidase has also been constructed in each of S60, PDG-1, and GAS-1.

[39] Samples of gellan made from strains GAS-1 (with a deletion of the gene for arylsulfatase) and GBAD-1 (with deletions of genes for both arylsulfatase and β-glucuronidase) were evaluated for p-cresol production at monthly intervals in an ultra-high temperature dairy application test. Gellan samples from the wild-type strain

produced 3 to 152 (average 65) ppb p-cresol after one month and 4 to 212 (average 96) ppb p-cresol after two months. Samples of gellan from GAS-1 produced about 1 to 3 ppb p-cresol after one to five months. Samples of gellan from GBAD-1 produced less than 1 ppb (limit of detection) when tested for up to three months.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

We Claim:

1. A gellan gum substantially free of catalytically active arylsulfatase protein and substantially free of any denaturing agent and substantially free of denatured arylsulfatase protein.

2. A gellan gum substantially free of catalytically active β-glucuronidase protein and substantially free of any denaturing agent and substantially free of denatured β-glucuronidase protein.

3. A gellan gum substantially free of catalytically active arylsulfatase and catalytically active β-glucuronidase proteins and substantially free of any denaturing agent and substantially free of denatured arylsulfatase and β-glucuronidase proteins.

4. A culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum of claim 1, wherein the *Sphingomonas elodea* is arylsulfatase deficient and comprises a deletion, insertion or inactivating mutation in all or part of an arylsulfatase gene.

5. A culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum of claim 2, wherein the *Sphingomonas elodea* is β-glucuronidase deficient and comprises a deletion, insertion or inactivating mutation in all or part of a β-glucuronidase gene.

6. A culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum of claim 3, wherein the *Sphingomonas elodea* is arylsulfatase deficient and is β-glucuronidase deficient and comprises a deletion, insertion or inactivating mutation in all or part of both an arylsulfatase gene and a β-glucuronidase gene.

7. A precipitated culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum of claim 1, wherein the *Sphingomonas elodea* is arylsulfatase deficient and comprises a deletion, insertion or inactivating mutation in all or part of an arylsulfatase gene.

8. A precipitated culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum of claim 2, wherein the *Sphingomonas elodea* is β-glucuronidase

deficient and comprises a deletion, insertion or inactivating mutation in all or part of a β-glucuronidase gene.

9. A precipitated culture broth of *Sphingomonas elodea* which is useful in production of the gellan gum of claim 3, wherein the *Sphingomonas elodea* is arylsulfatase deficient and is β-glucuronidase deficient and comprises a deletion, insertion or inactivating mutation in all or part of both an arylsulfatase gene and a β-glucuronidase gene.

10. A sterilized milk product comprising the gellan gum of any one of claims 1 to 3.

11. A method of producing a gellan gum comprising:

growing *Sphingomonas elodea* in a culture medium, wherein the *Sphingomonas elodea* produces no catalytically active arylsulfatase or no catalytically active β-glucuronidase or no catalytically active arylsulfatase and no catalytically active β-glucuronidase;

collecting the culture medium;

precipitating gellan gum from the culture medium.

12. The method of claim 11 further comprising:

adding the gellan gum to a dairy product.

13. A microbiologically pure culture of *Sphingomonas elodea* which is arylsulfatase deficient and which comprises a deletion, insertion or inactivating mutation in all or part of an arylsulfatase gene.

14. A microbiologically pure culture of *Sphingomonas elodea* which is β-glucuronidase deficient and which comprises a deletion, insertion or inactivating mutation in all or part of a β-glucuronidase gene.

15. A microbiologically pure culture of *Sphingomonas elodea* which is arylsulfatase deficient and β-glucuronidase deficient and which comprises a deletion, insertion or inactivating mutation in all or part of both an arylsulfatase gene and a β-glucuronidase gene.

16. A gellan gum substantially free of catalytically active arylsulfatase protein or catalytically active β-glucuronidase protein and substantially free of any denaturing

agent and substantially free of denatured arylsulfatase protein or β-glucuronidase protein, wherein the gellan gum is made by the microbiologically pure culture of any one of claims 13 to 15.

17. An isolated and purified polynucleotide encoding a *Sphingomonas elodea* arylsulfatase comprising an amino acid sequence according to SEQ ID NO: 2.

18. The isolated and purified polynucleotide according to claim 17 which comprises a nucleotide sequence according to nucleotides 521 to 2176 of SEQ ID NO: 1.

19. A method of producing isolated and purified mutated *Sphingomonas elodea* DNA which is useful in production of the gellan gum of claim 1, the method comprising:

producing a mutation in an arylsulfatase coding sequence of *Sphingomonas elodea* DNA, wherein the arylsulfatase coding sequence corresponds to SEQ ID NO: 1, wherein the mutation comprises a deletion of one or more of nucleotides 521 to 2176 of the arylsulfatase coding sequence, and wherein the mutation results in reduction or loss of arylsulfatase activity; and

isolating and purifying all or part of the mutated *Sphingomonas elodea* DNA.

20. The method of claim 19 wherein the isolated and purified mutated *Sphingomonas elodea* DNA comprises at least a portion of a sequence adjacent to and upstream of a start codon of an arylsulfatase gene ligated to a portion of a sequence adjacent to and downstream of a stop codon of the arylsulfatase gene.

21. A method of producing isolated and purified mutated *Sphingomonas elodea* DNA which is useful in production of the gellan gum of claim 2, the method comprising:

producing a mutation in a β-glucuronidase coding sequence of *Sphingomonas elodea* DNA, wherein the β-glucuronidase coding sequence corresponds to SEQ ID NO: 4, wherein the mutation comprises a deletion of one or more of nucleotides 1588 to 3447 of the β-glucuronidase coding sequence, and wherein the mutation results in reduction or loss of β-glucuronidase activity; and

isolating and purifying all or part of the mutated *Sphingomonas elodea* DNA.

22. The method of claim 21 wherein the isolated and purified mutated *Sphingomonas elodea* DNA comprises at least a portion of a sequence adjacent to and upstream of

a start codon of a β-glucuronidase gene ligated to a portion of a sequence adjacent to and downstream of a stop codon of the β-glucuronidase gene.

23. An isolated and purified mutated *Sphingomonas elodea* DNA produced by the method of any one of claims 19 to 22.

24. An isolated and purified polynucleotide which is useful in production of the gellan gum of claim 1, the isolated and purified polynucleotide comprising a nucleotide sequence corresponding to SEQ ID NO: 1 in which one or more of nucleotides 521 to 2176 have been deleted such that the isolated and purified polynucleotide encodes an inactive arylsulfatase or an arylsulfatase of reduced activity.

25. An isolated and purified polynucleotide which is useful in production of the gellan gum of claim 2, the isolated and purified polynucleotide comprising a nucleotide sequence corresponding to SEQ ID NO: 4 in which one or more of nucleotides 1588 to 3447 have been deleted such that the isolated and purified polynucleotide encodes an inactive β-glucuronidase or a β-glucuronidase of reduced activity.

26. A method of producing isolated and purified mutated *Sphingomonas elodea* DNA which is useful in production of the gellan gum of claim 3, the method comprising:

producing a mutation in an arylsulfatase coding sequence of *Sphingomonas elodea* DNA, wherein the arylsulfatase coding sequence corresponds to SEQ ID NO: 1, wherein the mutation comprises a deletion of one or more of nucleotides 521 to 2176 of the arylsulfatase coding sequence, and wherein the mutation results in reduction or loss of arylsulfatase activity;

producing a mutation in a β-glucuronidase coding sequence of *Sphingomonas elodea* DNA, wherein the β-glucuronidase coding sequence corresponds to SEQ ID NO: 4, wherein the mutation comprises a deletion of one or more of nucleotides 1588 to 3447 of the β-glucuronidase coding sequence, and wherein the mutation results in reduction or loss of β-glucuronidase activity; and

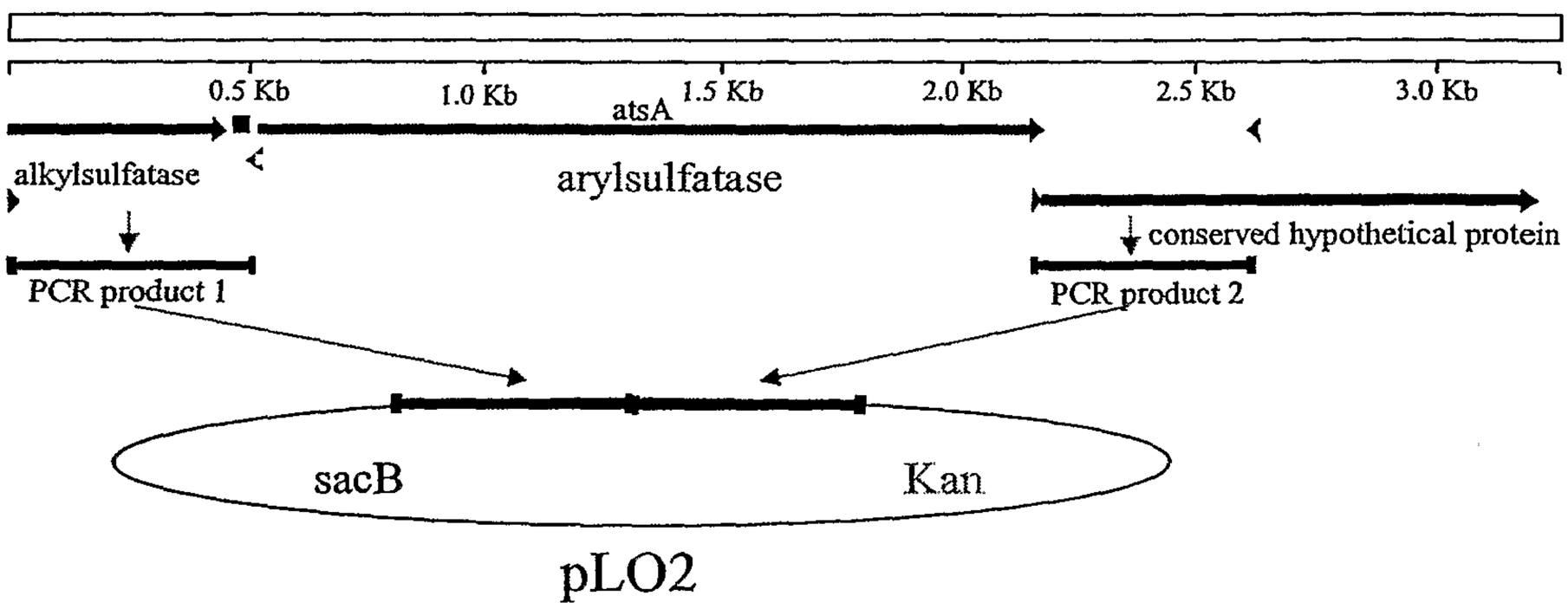
isolating and purifying all or part of the mutated *Sphingomonas elodea* DNA.

27. An isolated and purified polynucleotide which is useful in production of the gellan gum of claim 3, the isolated and purified polynucleotide comprising:

(i) a nucleotide sequence corresponding to SEQ ID NO: 1 in which one or more of nucleotides 521 to 2176 have been deleted such that the isolated and purified polynucleotide encodes an inactive arylsulfatase or an arylsulfatase of reduced activity; and

(ii) a nucleotide sequence corresponding to SEQ ID NO: 4 in which one or more of nucleotides 1588 to 3447 have been deleted such that the isolated and purified polynucleotide encodes an inactive β-glucuronidase or a β-glucuronidase of reduced activity.

Figure 1.


0.5 Kb
1.0 Kb
1.5 Kb
2.0 Kb
2.5 Kb
3.0 Kb
atsA
alkylsulfatase
arylsulfatase
conserved hypothetical protein
PCR product 1
PCR product 2
sacB
Kan
pLO2

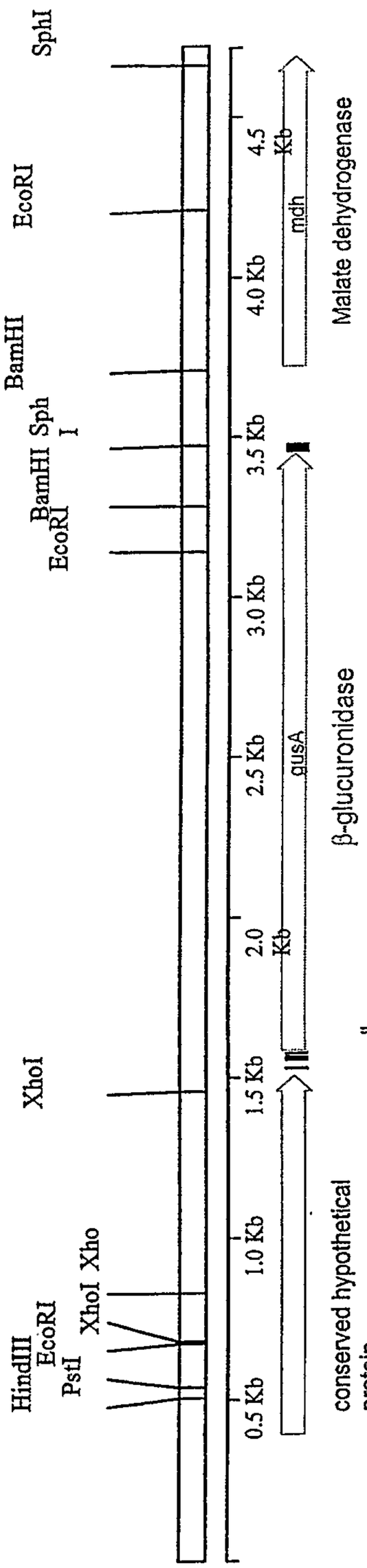
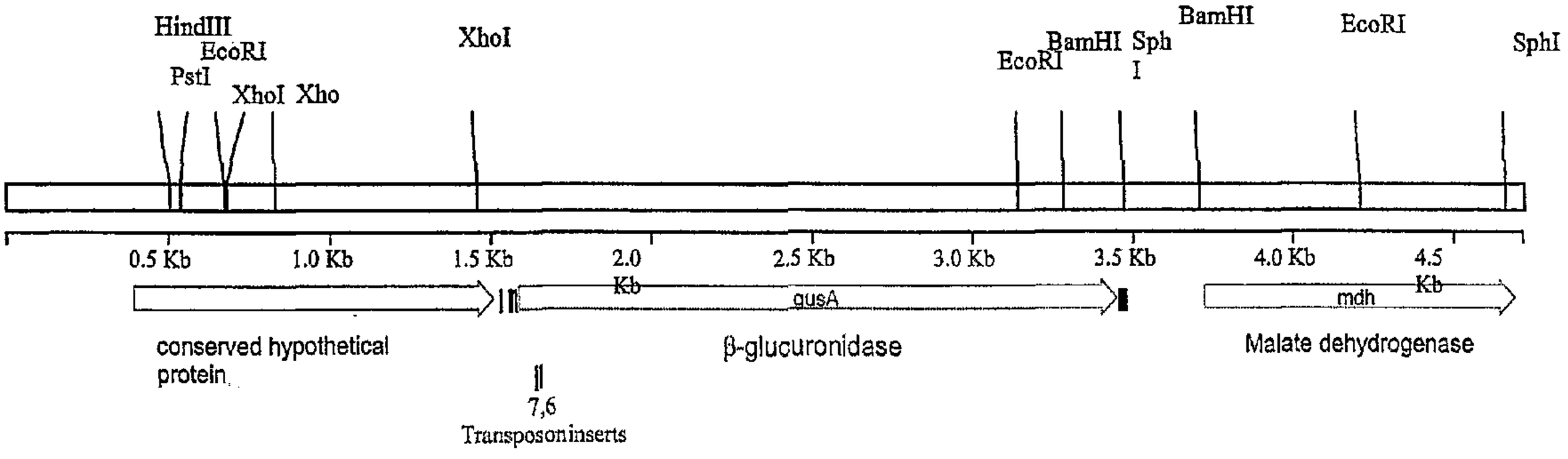

SphI
EcoRI
BamHI
BamHI Sph I
EcoRI
4.5 Kb
4.0 Kb
3.5 Kb
3.0 Kb
2.5 Kb
2.0 Kb
1.5 Kb
1.0 Kb
0.5 Kb
mdh
Malate dehydrogenase
gusA
β-glucuronidase
XhoI
HindIII
EcoRI
PstI
XhoI Xho
conserved hypothetical protein
7,6
Transposoninserts

Figure 2.


HindIII
EcoRI
PstI
XhoI
Xho
XhoI
EcoRI
BamHI
Sph
I
BamHI
EcoRI
SphI
0.5 Kb
1.0 Kb
1.5 Kb
2.0
Kb
2.5 Kb
3.0 Kb
3.5 Kb
4.0 Kb
4.5
Kb
gusA
mdh
conserved hypothetical
protein
β-glucuronidase
Malate dehydrogenase
7,6
Transposon inserts