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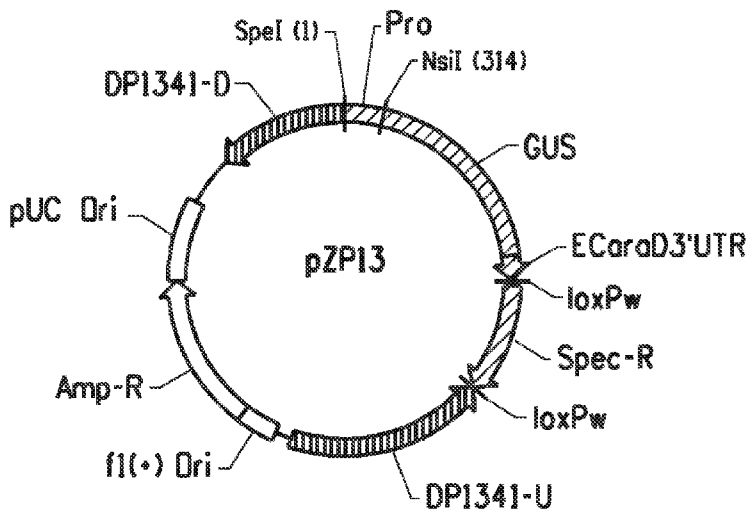


FIG. 1

(57) Abstract: Synthetic, derivative promoters for expression of chimeric genes in *Zymomonas*, *Zymobacter*, and related bacteria were created. The promoters have a C to T change in the nucleotide at position 90 of the *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene promoter. Promoters with this change have higher expression as compared to the native promoter from which they are derived and are useful for genetic engineering for expressing coding regions or regulatory RNA to obtain high levels of expressed proteins or regulatory RNAs.





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TITLE

## HIGH EXPRESSION ZYMOMONAS PROMOTERS

FIELD OF THE INVENTION

5 The invention relates to the fields of microbiology and genetic engineering. More specifically, new promoters for directing expression of chimeric genes in bacteria were identified.

BACKGROUND OF THE INVENTION

10 Production of ethanol by microorganisms provides an alternative energy source to fossil fuels and is therefore an important area of current research. *Zymomonas mobilis* is a natural ethanologen that can be used for commercial production of ethanol. However, *Z. mobilis* does not naturally utilize xylose, which is the major pentose in hydrolyzed  
15 lignocellulosic materials. It is desirable for an ethanologen to utilize xylose, to make full use of the carbohydrate substrates in lignocellulosic biomass hydrolysate.

*Zymomonas mobilis* and other bacterial ethanologens which do not naturally utilize xylose may be genetically engineered for xylose utilization  
20 by introduction of genes encoding 1) xylose isomerase, which catalyses the conversion of xylose to xylulose; 2) xylulokinase, which phosphorylates xylulose to form xylulose 5-phosphate; 3) transketolase; and 4) transaldolase. There has been success in engineering *Z. mobilis* strains for xylose metabolism (US 5,514,583, US 5,712,133, US  
25 6,566,107, US 5,726,053, Feldmann et al. (1992) Appl Microbiol Biotechnol 38: 354-361, Zhang et al. (1995) Science 267:240-243), as well as a *Zymobacter palmae* strain (Yanase et al. (2007) Appl. Environ. Microbiol. 73:2592-2599). However, typically the engineered strains do not grow and produce ethanol as well on xylose as on glucose.

30 For this engineering, genes encoding the heterologous proteins for xylose metabolism have been expressed from promoters that are active in *Z. mobilis* cells, typically the promoter of the *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene or the promoter of the *Z. mobilis* enolase gene. Strains engineered for xylose utilization have been adapted by

serial passage on xylose medium, resulting in strains with improved xylose utilization as described in US 7223575 and US 7741119. US7989206 and US7998722 disclose *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene promoters with improved activities and their use for  
5 expressing xylose isomerase for xylose utilization in *Zymomonas*.

There remains a need for additional strong promoters that may be used in genetic engineering of *Zymomonas*, and other bacterial ethanologens to express genes that confer improved xylose utilization. The promoters may be used for expression of other genes as well.

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### SUMMARY OF THE INVENTION

The present invention provides a synthetic promoter that is a derivative of the promoter of the *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene, which has improved activity.

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Accordingly, the invention provides a synthetic nucleic acid molecule comprising a *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene promoter that has a base substitution of T for C in position 90; wherein the position number is of SEQ ID NO:1. This based substitution has the effect of increasing the strength of the promoter as  
20 compared to a native *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene promoter.

In other aspects the invention provides a chimeric gene comprising a synthetic nucleic acid molecule having the above mentioned base substitution and a vector comprising the same.

25

In further aspects the invention provides a method of transforming a bacterial cell selected from the group consisting of *Zymomonas* cells and *Zymobacter* cells comprising introducing into the cell the synthetic nucleic acid molecule of the invention.

30

In yet another aspect the invention provides a recombinant bacterial strain selected from the group consisting of *Zymomonas* and *Zymobacter* comprising the synthetic nucleic acid molecule of the invention.

In another embodiment the invention provides a method for producing an improved synthetic glyceraldehyde-3-phosphate dehydrogenase gene promoter comprising:

- 5 a) isolating a nucleic acid fragment comprising a glyceraldehyde-3-phosphate dehydrogenase gene promoter region from a glyceraldehyde-3-phosphate dehydrogenase gene from *Zymomonas* or *Zymobacter*;
- 10 b) introducing in to the isolated fragment of a) a base substitution of T for C at position 90, thereby producing an improved synthetic glyceraldehyde-3-phosphate dehydrogenase gene promoter.

#### BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCE

##### DESCRIPTIONS

15 Figure 1 shows a plasmid map of pZP13.

The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions which  
20 form a part of this application.

The following sequences conform with 37 C.F.R. 1.821-1.825 (“Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules”) and are consistent with World Intellectual Property Organization (WIPO) Standard  
25 ST.25 (2009) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in  
37 C.F.R. §1.822.

30 SEQ ID NO:1 is the nucleotide sequence of the native *ZmPgap* of the CP4 strain of *Z. mobilis*. [#1 in 4236]

SEQ ID NO:2 is the nucleotide sequence of the *ZmPgap* of the CP4 strain of *Z. mobilis* with a change of C to T at position 90.

SEQ ID NO:3 is the nucleotide sequence of the native *ZmPgap* of the ZM4 strain of *Z. mobilis*. [#2 in 4236]

SEQ ID NO:4 is the nucleotide sequence of the *ZmPgap* of the ZM4 strain of *Z. mobilis* with a change of C to T at position 90.

5 SEQ ID NO:5 is the nucleotide sequence of the *ZmPgap* of pZB4 [T del #3 in 4236]

SEQ ID NO:6 is the nucleotide sequence of the *ZmPgap* of pZB4 with a change of C to T at position 90.

10 SEQ ID NO:7 is the nucleotide sequence of the *ZmPgap* of the ZM4 strain of *Z. mobilis* with the deletion of T at position 285 as in pZB4 and with a change of C to T at position 90.

SEQ ID NO:8 is the nucleotide sequence of the 1,951-bp DP1341-U fragment.

15 SEQ ID NO:9 is the nucleotide sequence of the 1,255-bp DP1341-D fragment.

SEQ ID NO:10 is the nucleotide sequence of the GUS coding sequence.

SEQ ID NO:11 is the nucleotide sequence of the *E. coli* *araD* 3' UTR.

20 SEQ ID NO:12 is the nucleotide sequence of the Spec-R expression cassette.

SEQ ID NO:13 is the nucleotide sequence of pZP1332.

SEQ ID NO:14 is the nucleotide sequence of pZP1337.

25 SEQ ID NO:15 is the nucleotide sequence of the loxPw fragment that is 5' to the Spec-R expression cassette.

SEQ ID NO:16 is the nucleotide sequence of the loxPw fragment that is 3' to the Spec-R expression cassette.

30 The flanking loxPw sequences, which include the loxPw sites and restriction sites, are SEQ ID NOs:15 and 16.

### DETAILED DESCRIPTION

The following definitions may be used for the interpretation of the claims and specification:

As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having,” “contains” or “containing,” or any other  
5 variation thereof, are intended to cover a non-exclusive inclusion. For example, a composition, a mixture, process, method, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent  
10 to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, “or” refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A  
15 and B are true (or present).

Also, the indefinite articles “a” and “an” preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances (i.e. occurrences) of the element or component. Therefore “a” or “an” should be read to include one or at least one, and the  
20 singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

The term “invention” or “present invention” as used herein is a non-limiting term and is not intended to refer to any single embodiment of the particular invention but encompasses all possible embodiments as  
25 described in the specification and the claims.

As used herein, the term “about” modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or use  
30 solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or carry out the methods; and the like. The term “about” also encompasses amounts that differ due to different

equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term "about" means within 10% of the reported numerical value, preferably within 5% of the reported numerical value.

The term "xylose isomerase" refers to an enzyme that catalyzes the interconversion of D-xylose and D-xylulose. Xylose isomerases (XI) belong to the group of enzymes classified as EC 5.3.1.5.

The term "xylulokinase" refers to an enzyme that phosphorylates xylulose to form xylulose 5-phosphate.

The terms "transketolase" and "transaldolase" refer to two enzymes of the pentose phosphate pathway which convert xylulose 5-phosphate to intermediates that couple pentose metabolism to the glycolytic Entner-Doudoroff pathway permitting the metabolism of xylose to ethanol.

The term "gene" refers to a nucleic acid fragment that expresses a specific protein or functional RNA molecule, which may optionally include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" or "wild type gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes.

The term "promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In

general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters".

10           The term "expression", as used herein, refers to the transcription and stable accumulation of coding (mRNA) or functional RNA derived from a gene. Expression may also refer to translation of mRNA into a polypeptide. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

15           The term "transformation" as used herein, refers to the transfer of a nucleic acid fragment into a host organism, resulting in genetically stable inheritance. The transferred nucleic acid may be in the form of a plasmid maintained in the host cell, or some transferred nucleic acid may be integrated into the genome of the host cell. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

20           The terms "plasmid" and "vector" as used herein, refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "selectable marker" means an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, i.e., resistance to an antibiotic, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid of interest.

As used herein the term "codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA.

The term "heterologous" means not naturally found in the location of interest. For example, a heterologous gene refers to a gene that is not naturally found in the host organism, but that is introduced into the host organism by gene transfer. For example, a heterologous nucleic acid molecule that is present in a chimeric gene is a nucleic acid molecule that is not naturally found associated with the other segments of the chimeric gene, such as the nucleic acid molecules having the coding region and

promoter segments not naturally being associated with each other.

As used herein, an “synthetic nucleic acid molecule” is a man made or manipulated polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. A  
5 synthetic nucleic acid molecule in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term “*Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene promoter” and “*ZmPgap*” refer to a nucleic acid molecule with  
10 promoter activity that has a nucleotide sequence that naturally occurs upstream of the glyceraldehyde-3-phosphate dehydrogenase coding region in the *Z. mobilis* genome. These terms refer to the promoters of strains of *Z. mobilis* such as the CP4 and ZM4 strains (SEQ ID NOs:1 and 2, respectively) and to variants in sequence and/or length that direct  
15 expression at a level that is not substantially different , such as the *ZmPgap* of pZB4 (SEQ ID NO:3).

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*,  
20 2<sup>nd</sup> ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1989 (hereinafter “Maniatis”); and by Silhavy, T. J., Bennis, M. L. and Enquist, L. W. *Experiments with Gene Fusions*; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; and by Ausubel, F. M. et al., In *Current Protocols in Molecular Biology*, published by Greene  
25 Publishing and Wiley-Interscience, 1987.

The present invention relates to synthetic, derivative promoters for expression of chimeric genes in *Zymomonas*, *Zymobacter*, and related bacteria. The promoters have higher expression as compared to the native promoter from which they are derived and are useful for genetic  
30 engineering for expressing coding regions or regulatory RNA in *Zymomonas*, *Zymobacter*, and related bacteria to obtain high levels of expressed proteins or regulatory RNAs. For example, the present promoters may be used in genetic engineering to obtain improved xylose

utilization by *Zymomonas* cells by expression of a coding region for an enzyme of a xylose utilization pathway, such as xylose isomerase.

#### Improved glyceraldehyde-3-phosphate dehydrogenase gene promoters

5           The present promoters are derivatives of the promoter from the *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene (*ZmPgap*) that have a substitution of T for C at position 90 in the *ZmPgap*, with position 90 referring to nucleotide +90 in SEQ ID NO:1. Promoters with this nucleotide change have increased activity as compared to the native  
10 *ZmPgap*.

          In one embodiment the present promoter has the nucleotide sequence of SEQ IN NO:2, which is identical to SEQ ID NO:1 with the exception of a T at position 90.

          In various embodiments, the present promoter is any *ZmPgap*  
15 having a substitution of T for C at position 90 in reference to the nucleotides of SEQ ID NO:1. The sequence of *ZmPgap* is not a single sequence, but may have some variation in sequence that has no substantial effect on promoter function. Having no substantial effect on promoter function means that the promoter sequence directs an  
20 expression level that is substantially similar to the level of expression directed by a *ZmPgap* present in a natural *Zymomonas mobilis* strain. Variation in sequence may naturally occur between different isolates or strains of *Zymomonas mobilis*, such as the difference between the *ZmPgap* of the CP4 and ZM4 strains at position 276 in reference to the  
25 nucleotides of SEQ ID NO:1, where in CP4 there is an A and in ZM4 there is a G (SEQ ID NOs:1 and 3, respectively. In one embodiment the present promoter has the sequence of the *ZmPgap* of the ZM4 strain with the substitution of T for C at position 90 (SEQ ID NO:4).

          In addition, there may be one or more insertion or deletion in the  
30 *ZmPgap* sequence which has no substantial effect on promoter function, such as exists in the pZB4 vector. The *ZmPgap* of the pZB4 vector is missing the T at position 285 in reference to the nucleotides of SEQ ID NO:1, giving SEQ ID NO:5. This promoter has an A at position 276 as in

the CP4 strain. In one embodiment the present promoter has the sequence of the *ZmPgap* of the pZB4 vector with the substitution of T for C at position 90 (SEQ ID NO:6).

5 With one or more insertion or deletion, position number with respect to SEQ ID NO:1 may be affected, depending on whether an insertion or deletion occurs between the 5' end of the promoter and what is position 90 in SEQ ID NO:1. Thus the position for the C to T change can best be recognized by the sequence context. The context for the C to T change is: CAGGGAC**C**GACAAT where the bold and underlined C is the nucleotide  
10 changed to T in the present derivative promoter. One skilled in the art will readily recognize a change from C to T, also called a substitution of T for C, at this nucleotide, which is called position 90 with respect to SEQ ID NO:1.

15 In another embodiment the present promoter has the substitution of T for C at position 90, the missing T at position 285 as in pZB4, and the G at position 276 as in the ZM4 strain (SEQ ID NO:7). A promoter with the substitution of T for C at position 90, the missing T at position 285 as in pZB4, and the A at position 276 as in the CP4 strain is the same as SEQ ID NO:6. All positions are with reference to SEQ ID NO:1.

20 Thus examples of the present promoter include SEQ ID NOs:2, 4, 6, and 7. A *ZmPgap* having a substitution of T for C at position 90, including in the context given above, are called herein *PgapU* or  $P_{gapU}$ .

#### Preparing an improved *PgapU*

25 The described substitution at position 90 may be introduced into a *ZmPgap* nucleic acid molecule by any method known to one skilled in the art. For example, an oligonucleotide having the mutation and surrounding DNA sequence may be synthesized and cloned into a larger promoter DNA fragment, substituting for a segment without the position 90  
30 substitution. Primers containing the nucleotide change and some adjacent promoter sequence may be synthesized and used in PCR to prepare the promoter fragment. An entire promoter DNA fragment may be synthesized

as multiple oligonucleotides that are ligated together. Site-directed mutagenesis may be used to introduce the nucleotide substitution.

Improved P<sub>gap</sub> in chimeric genes and vectors, introduction into bacterial cells

5 The present promoter may be operably linked to a heterologous nucleic molecule that is to be expressed in a bacterial cell, forming a chimeric nucleic acid molecule, or chimeric gene which is another aspect of the present invention. The designing and construction of chimeric genes  
10 are well known to one skilled in the art. A chimeric gene typically includes a promoter, a heterologous nucleic acid molecule to be expressed, and a 3' termination control region. Termination control regions may be derived from various genes, and are often taken from genes native to a target host cell. The operably linked heterologous nucleic acid molecule may be any  
15 nucleic acid molecule whose expression is desired in a bacterial cell, including, for example, a coding region for a protein or peptide, or a nucleic acid for expression of a functional RNA. Functional RNAs include, for example, antisense RNAs, ribozymes, and interfering RNAs. In addition an operon may be constructed that comprises the promoter  
20 described herein and multiple coding regions expressed from the promoter.

The promoters described herein may be used in chimeric genes for expression in bacteria belonging to *Zymomonas* or *Zymobacter*. The chimeric genes may be used for expression of any protein involved in  
25 production of a product of *Zymomonas* or *Zymobacter*. For example, one or more enzymes involved in synthesis of an amino acid such as alanine or of sorbitol or xylitol may be expressed from a chimeric gene having these promoters. The chimeric genes may be expressed in a natural *Zymomonas* or *Zymobacter* strain that does not utilize xylose, or in a  
30 xylose- utilizing strain. Also the promoters described herein may be used for expression of enzymes related to xylose metabolism or another metabolic pathway.

The chimeric genes described herein are typically constructed in or transferred to a vector for further manipulations. Vectors are well known in the art. Certain vectors are capable of replicating in a broad range of host bacteria and can be transferred by conjugation. The complete and annotated sequence of pRK404 and three related vectors: pRK437, pRK442, and pRK442(H) are available. These derivatives have proven to be valuable tools for genetic manipulation in gram-negative bacteria (Scott et al., *Plasmid* 50(1):74-79 (2003)).

Other well-known vectors may be used in different target host cells. Particularly useful for expression in *Zymomonas* are vectors that can replicate in both *E. coli* and *Zymomonas*, such as pZB188 which is described in US 5,514,583. Vectors may include plasmids for stable autonomous replication in a cell, and plasmids for carrying constructs to be integrated into bacterial genomes. Plasmids for DNA integration may include transposons, regions of nucleic acid sequence homologous to the target bacterial genome, or other sequences supporting integration. An additional type of vector may be a transposome produced using, for example, a system that is commercially available from EPICENTRE<sup>®</sup>. It is well known how to choose an appropriate vector for the desired target host and the desired function.

A promoter described herein may also be constructed in a vector without an operably linked nucleic acid molecule for expression, and integrated adjacent to an endogenous coding region to replace an endogenous promoter in a bacterial genome or to add a promoter, for example to a coding region within an operon. Chromosomal promoter replacements may be accomplished using methods such as described by Yuan et al (Metab. Eng. (2006) 8:79-90), and White et al. (Can. J. Microbiol. (2007) 53:56-62).

Vectors comprising a promoter described herein may be introduced into a bacterial cell by well known methods, such as using freeze-thaw transformation, calcium-mediated transformation, electroporation, or conjugation.

Expression of heterologous nucleic acid molecules using *PgapU*

Increased levels of chimeric gene expression may be obtained using an improved *Pgap* described herein. As shown in Example 3 herein, expression of a chimeric gene containing the present promoter operably  
5 linked to a coding region gave over 4-fold increased activity of the expressed protein in a cell extract, as compared to activity of the protein expressed from the native *Pgap* .

In various embodiments, one or more sequences encoding a protein that improves xylose or arabinose utilization may be expressed as  
10 part of a chimeric gene including the present promoter. Coding regions that may be expressed in a xylose utilization pathway include xylose isomerase, xylulokinase, transketolase, and transaldolase. These enzymes, their encoding sequences, and their participation in a xylose utilization pathway are well-known. In particular, any xylose isomerase as  
15 disclosed in US 7,998,722, which is incorporated herein by reference, is a xylose isomerase that may be expressed by operably linking its coding sequence to the present promoter.

In addition, for arabinose utilization, coding regions that may be expressed include L-arabinose isomerase to convert L-arabinose to L-  
20 ribulose, L-ribulokinase to convert L-ribulose to L-ribulose-5-phosphate, and L-ribulose-5-phosphate-4-epimerase to convert L-ribulose-5-phosphate to D-xylulose as disclosed in US 5,843,760, which is herein incorporated by reference. Additionally, sequences encoding other proteins that improve xylose utilization may be expressed, such as ribose-  
25 5-phosphate isomerase (RPI) which catalyzes the interconversion of ribulose-5-phosphate and ribose-5-phosphate and belongs to the group of enzymes classified as EC 5.3.1.6 (disclosed in US 20120156746, which is incorporated herein by reference) and ribulose-phosphate 3-epimerase (RPE) which catalyzes the interconversion of D-ribulose 5-phosphate and  
30 D-xylulose 5-phosphate and belongs to the group of enzymes classified as EC 5.1.3.1 (disclosed in US 20130157331, which is incorporated herein by reference).

In one embodiment the present promoter is used to express a heterologous nucleic molecule that encodes xylose isomerase for directing increased expression of xylose isomerase, as compared to expression from the *ZmPgap*. The improved *Pgap* and xylose isomerase coding region form a chimeric gene, which also generally includes a 3' termination control region. Termination control regions may be derived from various genes, and are often taken from genes native to a target host cell. The construction of chimeric genes is well known in the art.

Any xylose isomerase coding region may be used in a chimeric gene to express xylose isomerase from an improved *PgapU*. Xylose isomerase enzymes belong to the group EC5.3.1.5. Examples of suitable xylose isomerase proteins and encoding sequences that may be used are disclosed in US 7,998,722 and US 20110318801, which are incorporated herein by reference.

15

### EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

### GENERAL METHODS

The meaning of abbreviations is as follows: "kb" means kilobase(s), "bp" means base pairs, "nt" means nucleotide(s), "hr" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "L" means liter(s), "ml" or "mL" means milliliter(s), "μL" means microliter(s), "μg" means microgram(s), "ng" means nanogram(s), "mg" means milligram(s), "mM" means millimolar, "μM" means micromolar, "nm" means nanometer(s), "μmol" means micromole(s), "pmol" means picomole(s), "XI" is xylose isomerase, "nt" means nucleotide.

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Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (hereinafter “Maniatis”); and by Silhavy, T. J., Bennis, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and by Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987),

### Example 1

#### Construction of Promoter-GUS Test Integration Vectors

For integration of DNA in the *Zymomonas mobilis* genome, the pZP13 Double Cross Over (DCO) suicide vector was constructed. Figure 1 shows its schematic map. This plasmid has a pBluescript backbone, containing an *E. coli* replication site but no *Z. mobilis* replication site. Therefore, it cannot be propagated in *Z. mobilis*. Two fragments in pZP13 that target genomic recombination, DP1341-U and DP1341-D, were synthesized from *Z. mobilis* genomic DNA by PCR. Sequences referred to are in the *Z. mobilis* genome sequence: Seo et al. (2005) Nat. Biotechnol. 23:63-68; NCBI Reference: NC\_006526.2). The 1,951-bp DP1341-U fragment (SEQ ID NO:8) includes the first 217 bp (from nt-1 to nt-217) of the ZMO1250 coding sequence and 1,734-bp of upstream sequence. The 1,255-bp DP1341-D fragment (SEQ ID NO:9) includes the last 704 bp (from nt-218 to nt-921) of the ZMO1250 coding sequence and 551-bp of downstream sequence. These two fragments direct integration of transgenes located between them into the ZMO1250 coding sequence between nt-217 and nt-218 in the *Z. mobilis* genome. ZMO1250 is an open reading frame located in the *Z. mobilis* genomic sequence from 1,355,129 to 1,356,049, encoding a 306-aa hypothetical protein.

pZP13 was constructed to also include a chimeric GUS expression cassette located between the targeting fragments, which was used as a

reporter to determine the strength of a test promoter. It consists of a test promoter sequence (Pro) bounded by SpeI and NsiI sites, a 1,815-bp  $\beta$ -glucuronidase coding sequence (GUS; SEQ ID NO:10), and a 166-bp *E. coli araD* 3'UTR (ECaraD 3'UTR; SEQ ID NO:11). Located between the targeting fragments is also a 1,014-bp Spec-R expression cassette (SEQ ID NO:12) for selection of transformed *Z. mobilis* cells using spectinomycin, flanked by loxPw fragments. The flanking loxPw sequences, which include the loxPw sites and restriction sites, are SEQ ID NOs:15 and 16.

Test promoters used to express the GUS coding region were the *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase gene ( $P_{\text{gap}}$ ) and a derivative of this promoter,  $P_{\text{gapU}}$ .  $P_{\text{gap}}$  is a strong 305-bp glycolytic promoter (SEQ ID NO:1), which has been used previously for expressing of xylose-utilization genes in recombinant *Z. mobilis* strains.  $P_{\text{gapU}}$  is a 305-bp derivation of  $P_{\text{gap}}$  (SEQ ID NO:2) that has a change from C to T at position of 90 of the promoter sequence. The pZP13 suicide vectors containing these promoters driving expression of GUS were named pZP1332 containing  $P_{\text{gap}}$  (SEQ ID NO:13) and pZP1337 containing  $P_{\text{gapU}}$  (SEQ ID NO:14).

## Example 2

### Integration of Promoter-GUS Expression Cassettes

The test promoter-GUS chimeric genes were integrated into the genome of the wild type *Z. mobilis* strain ZW1 (ATCC #31821). Competent cells were prepared by growing ZW1 overnight in MRM3G5 (1% yeast extract, 15 mM  $\text{KH}_2\text{PO}_4$ , 4 mM  $\text{MgSO}_4$ , and 50 g/L glucose) at 30 °C with 150 rpm shaking. The  $\text{OD}_{600}$  value was measured using a Shimadzu UV-1200 Spectrophotometer (Kyoto, Japan). Cells were harvested and resuspended in fresh medium to an  $\text{OD}_{600}$  value of 0.05. Cells were grown under the same conditions to early-middle log phase ( $\text{OD}_{600}$  near 0.5), then harvested and washed twice with ice-cold water and once with ice-cold 10% glycerol. The resulting competent cells were collected and resuspended in ice-cold 10% glycerol to an  $\text{OD}_{600}$  value near 100. Since

transformation of *Z. mobilis* requires non-methylated DNA, pZP1332 and pZP1337 were first transformed into *E. coli* SCS110 competent cells (Stratagene, La Jolla, CA). For each transformation, one colony of transformed cells was grown in 10 mL LB-Amp100 (LB broth containing 100 mg/L ampicillin) overnight at 37 °C. DNA was prepared from each 10 mL-culture, using QIAprep Spin DNA Miniprep Kit (Qiagen, Valencia, CA).

Approximately 1 µg of non-methylated plasmid DNA was mixed with 50 µL of ZW1 competent cells in a 1 MM Electroporation Cuvette (VWR, West Chester, PA). The plasmid DNA was electroporated into the cells at 2.0 KV using a BT720 Transporater Plus (BTX-Genetronics, San Diego, CA). Transformed cells were recovered in 1 mL MMG5 medium (50g/L glucose, 10g/L yeast extract, 5 g/L tryptone, 2.5 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g/L K<sub>2</sub>HPO<sub>4</sub>, and 1 mM MgSO<sub>4</sub>) for 4 hours at 30 °C and grown on MMG5-Spec250 plate (MMG5 with 250 mg/L spectinomycin and 15 g/L agar) for 3 days at 30 °C, inside an anaerobic jar with an AnaeroPack (Mitsubishi Gas Chemical, New York, NY). The spectinomycin-resistant colonies were obtained and streaked onto a fresh MMG5-Spec250 plate, and grown under the same conditions as described above, indicating that the chimeric GUS/Spec-R transgene construct had been integrated into the genome of ZW1. To further confirm integration and expression of the GUS expression cassette, transformants were inoculated into 5 mL MRM3G5-Spec200 (MRM3G5 with 200 mg/L spectinomycin) in a 14 mL capped Falcon tube and grown overnight at 30 °C on a 150 rpm shaker. Cells of 100 µL cultures were spun down in a 1.5 mL microcentrifuge tube. The collected cells were washed with 1 mL 0.1M pH 7.0 phosphate buffer and then resuspended in 100 µL GUS staining solution (1 mL solution includes 500 µL 0.2 M sodium phosphate buffer, pH 7.0, 20 µL 0.5 M EDTA, pH8.0, 10 µL 10% triton X-100, 20 µL 50 mg/mL X-Glue dimethylformamide solution, 450 µL water). Color reactions were carried out by incubating the resuspension overnight at 37 °C. The reactions were stopped by removing the GUS solution and adding 100 µL of 70% EtOH. Many transformants had positive GUS staining as determined by

the signature blue color. Therefore, these transformants not only had integration of chimeric GUS/Spec-R construct, but also expression of both GUS and Spec-R transgenes. These transformants were named as ZW1-ZP1332 and ZW1-ZP1337.

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### Example 3

#### Promoter Comparison by GUS Activity Assay

To measure GUS activity, two transformants containing ZW1-ZP1332 (ZW1-ZP1332 #11 and #12) or ZW1-ZP1337 (ZW1-ZP1337 #1 and #8) were grown overnight in 2 mL MRM3G5-Spec200 at 30 °C with 150 rpm shaking. Then 50  $\mu$ L cultures were added into 5 mL fresh MRM3G5-Spec200 and grown 16 hrs at 30 °C with 150 rpm shaking in a 14 mL capped Falcon tube, until OD<sub>600</sub> was near 6. Cells were collected by centrifuge, washed with 1 mL GUS assay base buffer (50 mM NaPO<sub>4</sub> buffer, pH7.0, 1 mM EDTA) twice, resuspended in 1 mL protein extract buffer (GUS assay base buffer containing 5% glycerol). Each cell suspension was placed in a BIO101 2 mL capped tube containing Lysing Matrix A beads (BIO101, La Jolla, CA) and shaken 3 times on a BIO101 Fastprep FP1200 at Step 5 for 20 second. Cell debris and beads were spun down in a micro-centrifuge, and the supernatant was collected as protein extract.

Protein concentration in the extract was determined using Pierce Coomassie Protein Assay Solution (Thermo Fisher Scientific Inc., Rockford, IL). In the assay, 10  $\mu$ L standard BSA solutions, including 0.025, 0.05, 0.1, and 0.2  $\mu$ g/ $\mu$ L, as well as properly diluted protein extracts, were mixed with 200  $\mu$ L Protein Assay Solution in a 96-well plate and incubated for 5 min at room temperature. OD<sub>595</sub> was measured on a Victor III (Perkin Elmer, Waltham, MA) and protein concentration was calculated by the instrument according to the resultant standard curve.

Table 1 is a summary of protein concentrations in all extracts.

Table1. Protein concentrations in cell extracts

Promoter	ZW1-ZP Strain	Protein Concentration (ng/ $\mu$ L)
$P_{gap}$	1332-11	798
	1332-12	539
$P_{gapU}$	1337-1	705
	1337-8	1,000

A protocol for GUS activity assay was adapted from GUS Protocols: Using the GUS as a Reporter of Gene Expression (Ed. Sean R. Gallagher, Academic Press, Inc., 1992). It was modified to perform in a 96-well plate. In the assay, the ZW1-ZP1332 #11 and #12 protein extracts were diluted 20 times and the ZW1-ZP1337 #1 and #8 protein extracts were diluted 80 times using the protein extract buffer (GUS assay base buffer with 5% glycerol). Fresh GUS assay working buffer was prepared by mixing 3,930  $\mu$ L GUS assay base buffer, 20  $\mu$ L 1 M DTT, and 50  $\mu$ L 100 mM p-Nitrophenyl- $\beta$ -D-glucuronide (PNPG) and pre-warmed to 37  $^{\circ}$ C. A Falcon 96-well flat bottom plate was also prepared by adding 200  $\mu$ L 0.4 M  $Na_2CO_3$  stop solution to each well. A GUS assay reaction was assembled in a 0.2 mL PCR tube by adding 25  $\mu$ L diluted protein extract and 100  $\mu$ L pre-warmed GUS working buffer, and then incubated at 37  $^{\circ}$ C. At 15, 30, 45, and 60 min, 25  $\mu$ L of reaction was removed from the reaction tube and immediately mixed with 200  $\mu$ L stop solution in the prepared 96-well plate. Three parallel reactions were carried out for each protein extract. A mixture of 20  $\mu$ L GUS assay working buffer and 5  $\mu$ L protein extract buffer was added to a well as a blank. After completion of reaction, the 96-well plate was scanned on a Victor III plate reader to determine  $OD_{405}$  in each well. Reading of the blank was deducted from each reaction. Average  $OD_{405}$ , which reflects the release of 4-Nitrophenol by  $\beta$ -glucuronidase, was plotted against reaction time, resulting in a slope S ( $OD_{405}/min$ ) that indicates change of  $OD_{405}$  per minute. Finally, reaction rate R ( $OD_{405}/ug$  protein/min) was calculated based on the reaction slope S and protein amount added into reaction. Reaction slope (S) and rate (R) of each

protein extract are given in Table 2 below. Average reaction rates were used to calculate relative activities, which indicate promoter strength relative to  $P_{gap}$ . The result demonstrated that the  $P_{gapU}$  promoter had strength of approximately 4.4 fold higher than the  $P_{gap}$  promoter.

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Table 2. Relative promoter strength of  $P_{gapU}$  and  $P_{gap}$ .

Promoter	ZW1-ZP strain	S (OD <sub>405</sub> /min)	R (OD <sub>405</sub> /μg prot. /min)	Relative activity
$P_{gap}$	1332-11	0.0065	0.033	100%
	1332-12	0.0052	0.039	
$P_{gapU}$	1337-1	0.0065	0.144	442%
	1337-8	0.0113	0.174	

CLAIMS

What is claimed is:

1. A synthetic nucleic acid molecule comprising a *Z. mobilis*  
5 glycerinaldehyde-3-phosphate dehydrogenase gene promoter having a  
base substitution of T for C in position 90 of the native *Z. mobilis*  
glycerinaldehyde-3-phosphate dehydrogenase gene promoter, whose  
sequence is set forth in SEQ ID 1.
- 10 2. A synthetic nucleic acid molecule comprising a *Z. mobilis*  
glycerinaldehyde-3-phosphate dehydrogenase gene promoter sequence  
selected from the group consisting of SEQ ID NOs:2, 4, 6, and 7.
3. A chimeric gene comprising the synthetic nucleic acid molecule of  
15 claims 1 or 2 operably linked to a heterologous nucleic acid molecule.
4. The chimeric gene of claim 3 wherein the heterologous nucleic acid  
molecule encodes a protein or peptide.
- 20 5. The chimeric gene of claim 3 wherein the heterologous nucleic acid  
molecule codes for a regulatory RNA molecule selected from the group  
consisting of an antisense RNA, a ribozyme, and an interfering RNA.
6. A vector comprising the synthetic nucleic acid molecule of claims 1  
25 or 2.
7. A method of transforming a bacterial cell selected from the group  
consisting of *Zymomonas cells* and *Zymobacter cells* comprising  
introducing into the cell the synthetic nucleic acid molecule of claims 1 or  
30 2.
8. A method according to claim 7 wherein introducing comprises  
integrating the synthetic nucleic acid molecule of claims 1 or 2 into the

genome of the cell or maintaining on a stably replicating plasmid within the cell.

9. A recombinant bacterial strain comprising the synthetic nucleic acid  
5 of claims 1 or 2 wherein the bacterial strain is selected from the group consisting of *Zymomonas* and *Zymobacter*

10. The recombinant bacterial strain of claim 9 wherein said isolated nucleic acid molecule is the promoter of a chimeric gene.

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11. The recombinant bacterial strain of claim 10 wherein the chimeric gene comprises an isolated nucleic acid molecule encoding an enzyme selected from the group consisting of xylose isomerase, xylulokinase, transketolase, transaldolase, L-arabinose isomerase, L-ribulokinase, L-  
15 ribulose-5-phosphate-4-epimerase, ribose-5-phosphate isomerase, and ribulose-phosphate 3-epimerase.

12. A method for producing an improved synthetic glyceraldehyde-3-phosphate dehydrogenase gene promoter comprising:

20

a) isolating a nucleic acid fragment comprising a glyceraldehyde-3-phosphate dehydrogenase gene promoter region from a glyceraldehyde-3-phosphate dehydrogenase gene from *Zymomonas* or *Zymobacter*; and

25

b) introducing in to the isolated fragment of a) a base substitution of T for C at position 90, thereby producing an improved synthetic glyceraldehyde-3-phosphate dehydrogenase gene promoter.

13. An improved synthetic glyceraldehyde-3-phosphate dehydrogenase gene promoter made by the process of Claim 12.

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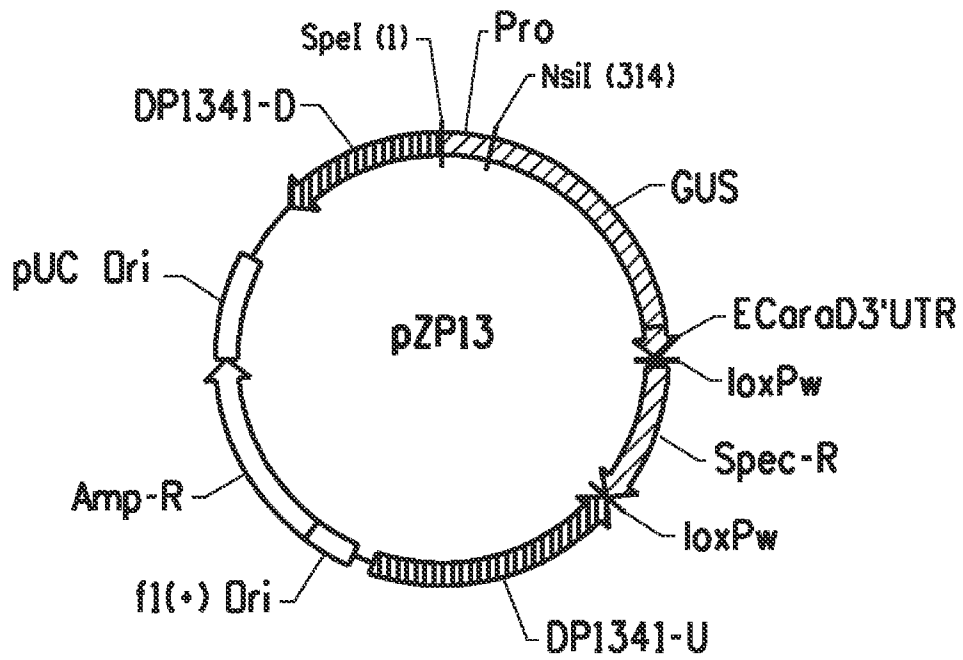


FIG. 1

# INTERNATIONAL SEARCH REPORT

International application No PCT/US2014/057386
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**A. CLASSIFICATION OF SUBJECT MATTER**  
 INV. C12N1/22 C12P7/10  
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, FSTA, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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Further documents are listed in the continuation of Box C.       See patent family annex.

\* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>
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Date of the actual completion of the international search  <p style="text-align: center; font-size: 1.2em;">15 January 2015</p>	Date of mailing of the international search report  <p style="text-align: center; font-size: 1.2em;">26/01/2015</p>
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer   <p style="text-align: center; font-size: 1.2em;">Bonello, Steve</p>
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International application No

PCT/US2014/057386

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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