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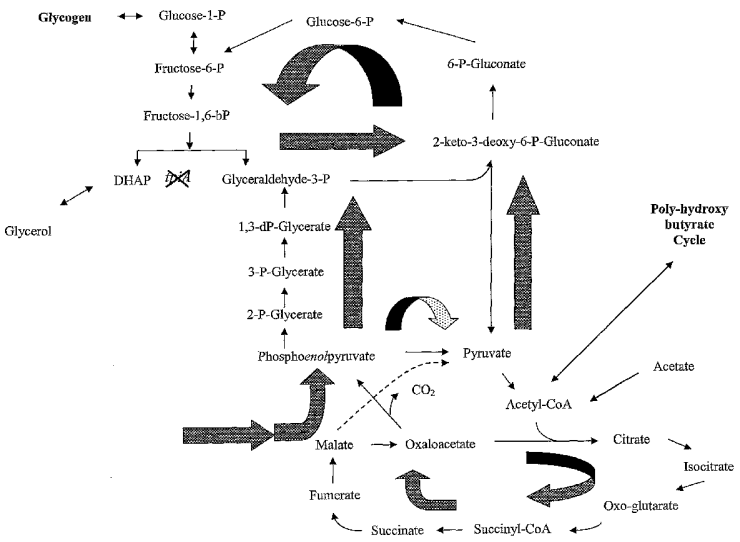
(54) **Title:** TRIOSE-PHOSPHATE ISOMERASE MUTANTS OF RHIZOBIAL BACTERIA




(57) **Abstract:** A method for increasing dry matter production by a selected nitrogen-fixing legume. The method comprises selecting a rhizobial bacterial culture capable of infecting and establishing an effective symbiotic nitrogen-fixing relationship with said legume, and manipulating the culture to impair and/or inactivate therein triose-phosphate isomerase enzyme activity. The culture may be genetically manipulated by inserting into the rhizobial bacterial DNA, at least one nucleotide sequence configured to interfere with the synthesis and/or activation and/or functional activity of a triose-phosphate isomerase enzyme. Compositions for inoculating legumes comprising rhizobial bacterial cultures manipulated to inactivate therein triose-phosphate isomerase enzyme activity. A biologically pure rhizobial bacterial culture, said culture manipulated to impair and/or inactivate therein triose-phosphate isomerase enzyme activity.

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# TRIOSE-PHOSPHATE ISOMERASE MUTANTS OF RHIZOBIAL BACTERIA

## TECHNICAL FIELD

The present invention relates to nitrogen fixation by legumes, and more particularly, to new mutants of rhizobial bacteria which increase the amount of dry matter formed by nitrogen-fixing legumes.

## BACKGROUND ART

Nitrogen fixation is a mutually beneficial symbiotic relationship between rhizobial bacteria and leguminous plants that occurs within specialized root-associated structures called nodules containing one or more bacteroids wherein the plants provide carbon substrates derived from photosynthesis, as sources of energy during their catabolic metabolism to drive rhizobial respiration and metabolism in the bacteroids during nitrogen fixation. Furthermore, the carbon intermediates formed during catabolism serve as key building blocks for synthesis of organic compounds that incorporate the nitrogen derived from fixation.

It is well-known that the nitrogen fixation processes mediated by rhizobial bacteria have very high requirements for energy in the form of ATP for the reduction of atmospheric nitrogen into ammonium ions by bacteroid nitrogenase enzymes. The ATP requirements are supplied through the flow of carbohydrates from photosynthate which is translocated to the root nodules. There, the photosynthate flows through several interconnected metabolic pathways which generally cleave disaccharides into 6-carbon monosaccharides which are then phosphorylated and subsequently catabolized through glycolytic processes, to produce C-4 dicarboxylic acid substrates that are supplied to the bactereoid as the sources of carbon and energy.

Within the bacteroid, the dicarboxylalic acid substrates must concurrently be: (a) oxidized by the TCA cycle to ultimately generate ATP, and (b)

metabolized gluconeogenically to provide carbon intermediates for bacteroid proliferation.

The directionality of carbon flow through these pathways is precisely controlled by specific enzymes at each reaction step. Each enzyme's activity is directly affected by the accumulation and/or disappearance of its intermediate substrates. Furthermore, many of the enzymes involved in these pathways can directly affect their individual mediated reactions in both anabolic and catabolic directions, and thereby cause synthesis of short-term storage products such as glycogen and poly-hydroxybutyrate when intermediate substrates accumulate in one or more of these pathways, or alternatively, cause catabolism of the short-term storage products to drive the pathways when intermediate substrates become limiting. For example, if intermediate carbon substrates accumulate in the TCA cycle and/or during the metabolism of malate into pyruvate, then the intermediate substrates are incorporated into a gluconeogenesis pathway which as a key step, synthesizes glyceraldehyde-3-phosphate (G3P) as an intermediate and incorporates it into fructose-1,6-bisphosphate (F-1,6-BP) for subsequent production of glycogen. For the most part, gluconeogenesis uses the same enzymes involved in glycolysis.

The interrelationships between carbon and nitrogen metabolic pathways in nitrogen-fixing legumes are complex and have not yet been clearly defined. It is known that under certain conditions, lower rates of photosynthesis may limit rates of nitrogen fixation due to reduced supply of carbon substrates for generation of ATP to drive nitrogen fixation, and also, may negatively affect the amount of nitrogenase enzyme produced within the bacteroids. However, it has been shown that legume nodules typically do not have excess nitrogenase enzyme capacity available to respond to significantly increased supply of photosynthates by significantly increasing the amounts of nitrogen fixed. Furthermore, it appears that nitrogen fixation is not generally limited by the supply of photosynthates but rather, by the flow and utilization within the nodules of carbon substrates derived from catabolism of photosynthates.

DISCLOSURE OF THE INVENTION

The exemplary embodiments of the present invention, at least in preferred forms, are directed to rhizobial bacteria for affecting nitrogen fixation by leguminous plants thereby increasing the amount of dry matter accumulating therein.

According to one aspect of the present invention, there is provided a rhizobial bacterium wherein said bacterium is lacking a functioning triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

According to another aspect of the present invention, there is provided a rhizobial bacterium wherein said bacterium is lacking the requisite genetic information for affecting functional triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

According to another aspect of the present invention, there is provided a rhizobial bacterium wherein said bacterium is lacking the requisite genetic information for metabolic synthesis and operation of functional triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

According to a preferred aspect of the present invention, there is provided a method for affecting a rhizobial bacterium capable of affecting nitrogen fixation in leguminous plants, whereby a functioning triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is inactivated.

In a preferred form, the invention provides a method for affecting a rhizobial bacterium capable of affecting nitrogen fixation in leguminous plants, whereby the requisite genetic information for metabolic synthesis and operation of functional triose-phosphate isomerase enzyme activity for conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate, is inactivated.

In another preferred form, the invention provides a method for affecting a rhizobial bacterium whereby the requisite genetic information for metabolic synthesis and operation of functional triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is removed from said bacterium.

According to a preferred aspect of the present invention, there is provided a *Sinorhizobium meliloti* bacterium capable of affecting nitrogen fixation in leguminous plants, whereby functional triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in said bacterium, is inactivated.

According to yet another preferred aspect of the invention, there is provided a method for affecting a *Sinorhizobium meliloti* bacterium capable of affecting nitrogen fixation in leguminous plants, whereby the requisite genetic information for metabolic synthesis and operation of functional triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is inactivated.

In a preferred form, the invention provides a method for affecting a *Sinorhizobium meliloti* bacterium whereby the requisite genetic information for metabolic synthesis and operation of functional triose-phosphate isomerase enzyme activity for interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is removed from said bacterium.

According to a further preferred aspect of the invention, there is provided a composition consisting of rhizobial bacteria wherein functional triose-phosphate isomerase enzyme activity is inactivated, said bacteria capable of affecting nitrogen fixation in leguminous plants, and a carrier.

In a preferred form, the invention provides a composition consisting of *Sinorhizobium meliloti* bacteria wherein functional triose-phosphate isomerase enzyme activity is inactivated, said bacteria capable of affecting nitrogen fixation in leguminous plants, and a carrier.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be described in conjunction with reference to the following drawings in which:

Fig. 1 is a prior art flow chart for glycolysis by rhizobial bacteria;

Fig. 2 is a prior art flow chart for gluconeogenesis by rhizobial bacteria; and

Fig.3 is a flow chart showing carbon pathways utilized by the rhizobial mutants of the present invention;

Fig. 4 shows the results of a BLASTN analysis of a section of DNA immediately flanking the Tn5 insert from a *Sinorhizobium meliloti tpiA* mutant; and

Fig. 5 is a map showing the region of the *Sinorhizobium meliloti* genome that contains *tpiA1* and the position of the Tn*5* insertion based on the BLASTN analysis.

## BEST MODES FOR CARRYING OUT THE INVENTION

Carbon flow in rhizobial bacteria provides reducing power in the form of (NAD(P)H), energy in the form of ATP (derived from oxidative phosphorylation), and carbon skeletons for the formation of precursor molecules needed for bacterial growth. Regardless of environmental conditions and physiological state, sustained delivery of these products is required for ongoing microbial growth and function. There are two general modes of operation for carbon flow and metabolism in rhizobial bacteria. These are (a) the glycolysis pathway which is based on metabolism of glucose, and (b) the gluconeogenesis pathway which is based on metabolism of carbon substrates which possess fewer than 6 carbon units. Each pathway will be discussed in detail to provide background for description of the present invention.

**Glycolysis pathway**

The catabolism of glucose in rhizobial bacteria, proceeds predominantly through the Entner-Doudoroff (ED) pathway as shown in Fig. 1 wherein glucose is phosphorylated to form glucose-6-phosphate which is subsequently converted to 6-phospho-gluconate (6PG) through reduction of $NADP^+$ to NADPH. The 6PG is then dehydrated to form a key 6-carbon intermediate in the ED pathway, 2-keto-3-deoxy-6-phosphogluconate (KDPG). KDPG is then catabolized by KDPG aldolase to form two 3-carbon intermediates (a) pyruvate, and (b) glyceraldehyde-3-phosphate (G3P). The pyruvate enters the TCA cycle to ultimately yield ATP, NADPH and NADH. Depending on the rate of pyruvate uptake into the TCA cycle and the subsequent accumulation and utilization of TCA intermediates, the G3P produced by KDPG catabolism may: (a) be further catabolized into pyruvate through phosphoenolpyruvate (PEP) via the lower half of the Embden-Meyerhof-Parnas pathway (EMP), or alternatively, (b) be recycled back into glucose-6-phosphate as shown by Portais et al. (1999, Eur. J. Biochem. 265: 473-480) and Gosselin et al. (FEBS Lett. 499: 45-49). This recycling of G3P is facilitated by its conversion to dihydroxyacetone phosphate (DHAP) mediated by triose-phosphate isomerase (*tpiA*) after which, the DHAP is condensed with G3P to form fructose-1,6-bisphosphate (F-1,6-BP) which is then converted to fructose-6-phosphate and then to glucose-6-phosphate. The anabolic formation of glucose-1-phosphate from G3P is essential because it is the beginning of a pathway that leads to the formation of glycogen, an important short-term storage product as well as a requisite intermediate in the synthesis of exo- and lipo-polysaccharides (EPS and LPS respectively).

**Gluconeogenesis pathway**

Free-living rhizobial bacteria can via the TCA cycle and gluconeogenesis pathway, concurrently assimilate and catabolize two different carbon storage compounds i.e. (a) poly-hydroxybutyrate, and (b) glycogen, as shown in Fig. 2 and discussed by Poole (2003, Crit. Rev. Plant Sci. 22: 37-78). Finan et al. (1988, J. Bacteriol. 170: 3396-3403) using genetic and biochemical analysis,

demonstrated that carbon flow in an anabolic direction from PEP to G3P in the lower portion of the EMP pathway, is absolutely essential for efficient gluconeogenesis in rhizobial bacteria. The G3P thus anabolically synthesized, may be further metabolized in one of two possible reactions. In the first reaction, some of the G3P is converted to DHAP through the activity of *tpiA*, following which the DHAP thus formed is condensed with additional G3P through the enzyme activity of F-1,6-BP aldolase to make F-1,6-BP. The second route is the anabolic reaction mediated by 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase to combine pyruvate and G3P into the 6-carbon KDGP. Finan et al. (1988) and Finan et al. (1991,Mol. Plant Microbe Interact. 18: 386-392) also demonstrated that the enzyme activities in the lower half of the EMP pathway flowing anabolically from PEP to G3P and also the enzyme activities required for synthesis of F-1,6-BP and fructose -6-phosphate, are not affected when rhizobial bacteria are grown on media containing gluconeogenic substrates such as pyruvate, malate, succinate, oxaloacetate and PEP, thereby indicating that the predominant gluconeogenic carbon flow in rhizobial bacteria must occur via the EMP pathway and not the ED pathway.

**Carbon flow in triose-phosphate isomerase rhizobial mutants of the present invention.**

Since it is known that rhizobial bacterial growth occurs on laboratory media containing carbon substrates with fewer than 6 carbon units, then it follows that *in planta*, if nitrogen-fixing bacteroids are not supplied with carbon substrates that can be catabolized into hexoses and converted to phosphorylated glucose intermediates, then these bacteroids must possess functioning gluconeogenic pathways.

Based on the genome sequence of *S. meliloti* published by Galilbert et al. (2001, Science 293: 668-672), there are only two possible pathways for gluconeogenesis, i.e., (1) the F-1,6BP aldolase-mediated synthesis of F-1,6BP from G3P and DHAP, and (2) the KDGP aldolase-mediated synthesis of KDGP from G3P and pyruvate. If *S. meliloti* is unable to use one or both these pathways,

these rhizobial bacteria would not be able synthesize LPS, EPS, or glycerol thereby leading to lethal phenotypes if they are grown on gluconeogenic substrates such as succinate or malate as shown by Finan et al. (1988).

It is known, based on information disclosed in reviews by Stowers (1985, Annu. Rev. Microbiol. 39: 89-108), Vance et al. (1991, Annu. Rev. Plant Physiol.Plant Mol. Biol. 42:373-392), and Lodwig et al. (Crit. Rev. Plant Sci. 22: 37-78) that the predominant carbon substrate supplied by leguminous plants to bacteroids is a C-4 dicarboxylic acid. Very little is known about how carbon compounds other than glucose and succinate are utilized by rhizobial bacteria.

Because of this dearth of information, we have been working to precisely clarify the carbon pathways in rhizobial bacteria using *Sinorhizobium meliloti* strain Rm 1021 as our model organism.

Our general approach was to generate large numbers of mutants of *S. meliloti* Rm 1021 using procedures described in more detail in the following Example Section, and then screen the mutants individually for growth on a variety of media, each containing a single carbon substrate, also described in more detail in the Example Section.

We surprisingly found a *S. meliloti* Rm 1021 mutant lacking functional *tpiA* enzyme activities (referred to hereinafter as *tpiA*$^-$ mutants) as evidenced by a lack of growth when cultured on either rhamnose or glycerol (both of which are gluconeogenic substrates). Since *tpiA*$^-$ mutants are capable of synthesizing LPS, EPS and glycerol, it is apparent that carbon flow in these mutants must flow through the KDGP aldolase-mediated synthesis of KDGP from G3P and pyruvate, i.e., the anabolic route of the ED pathway. Finan et al. (1991) demonstrated that enzyme activities when the ED pathway is flowing in the anabolic direction, are reduced 30-fold when rhizobial bacteria are grown on media containing dicarboxylic acid as a carbon source and also in *ex planta* bacteroids.

However, we surprisingly found that alfalfa inoculated with these *tpiA*$^-$ mutants accumulated significantly more dry matter than alfalfa inoculated with

the wild-type *S. meliloti* Rm 1021 strain. Therefore, it appears that in these *tpiA*⁻ mutants, gluconeogenesis is significantly impaired but not eliminated thereby causing an accumulation of carbon substrates associated with the lower half of the EMP pathway. These carbon intermediates are then available to be funnelled to other pathways. The most active sink for these "excess" carbon intermediates is the TCA cycle where they will be oxidized thereby generating reductant in the form of NAD(P)H that can be utilized for either oxidative phosphorylation thereby generating ATP, or alternatively, in combination with nitrogenase synthesis and activity in the bacteroids, thereby resulting increased nitrogen fixation and its incorporation into plant biomass.

The generation of rhizobial *tpiA*⁻ mutants, their characterization, and use for increasing the accumulation of dry matter in leguminous plants are described in more detail in the following examples.

## EXAMPLES

### BACTERIAL STRAINS, PLASMIDS MEDIA AND CULTURE CONDITIONS.

The S*inorhizobium meliloti* strains used and generated in these examples are listed in Table 1.

Table 1: *Sinorhizobium meliloti* strains.

| Strain | Relevant Characteristics |
|---|---|
| Rm1021 | Streptomycin resistant wild type (SU47 *str-21*) |
| Rm5000 | Rifampicin resistant wild-type (SU47 *rif-5*) |
| SRmA185 | Rm1021 *tpiA1*::Tn5 |
| SRmA327 | Rm1021 *tpiA1*::Tn5 transduced strain |
| SRmA355 | Rm5000 *tpiB1*::pKNOCK-Gm |
| SRmA366 | Rm1021 *tpiA1*::Tn5 *tpiB1*::pKNOCK-Gm |

Other bacterial strains and plasmids used and generated in these examples are listed in Table 2.

Table 2: Other bacterial strains and plasmids used in the examples.

| Strain or Plasmid | Relevant characteristics | Source |
|---|---|---|
| *Escherichia coli* | | |
| MM294A | *pro-82 thi-1 hsdR17 supE44* | lab collection |
| MT607 | MM294A *recA56* | lab collection |
| MT616 | MT607(pRK600) | lab collection |
| MT620 | MT607(pRK600::ΩTn5B20) | lab collection |
| DH5α | *endA hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 ϕ80dlacZΔM15* | lab collection |
| S17-1 | *recA* derivative of MM294Awith RP4-2 (Tc::Mu::Km::Tn7) integrated into the chromosome | lab collection |
| Plasmids | | |
| pBlueScript II SK | Cloning vector, ColE1 *oriV*, $Ap^r$ | Stratagene |
| pKNOCK-GM | Suicide vector for insertional mutagenesis R6K *ori*, RP4 *orit*, $Tc^r$ | Alexyev (1999, Biotechniques) 26:824-828 |
| pRK600 | pRK2013*npt* ::Tn( $Cm^r$ Nm-$Km^s$ | lab collection |
| pNP149 | pKNOCK-GM with 430 bp internal fbaB fragment cloned with *Bam*HI/*Kpn*I | this work |
| pNP150 | pKNOCK-GM with 3500 bp internal *tpiB* fragment cloned with *Bam*HI/*Kpn*I | this work |
| pNP151 | pBlueScript with 350 bp internal *tpiB* fragment cloned with *Bam*HI/*Kpn*I | this work |

The bacterial strains were routinely grown at 30°C on complex (LB) or defined (VMM) media as described by Sambrook et al. (1989, Molecular Cloning Manual, 2nd Ed., Cold Spring Harbour Laboratory, Cold Spring Harbour, NY) and Richardson et al. (2004, J. Bacteriol. 186:8433-8442). When required, antibiotics were used at the following concentrations: tetracycline (Tc) either 5 or 10 $\mu g\ ml^{-1}$; neomycin (Nm), 200 $\mu g\ ml^{-1}$; kanamycin (Kan), 50 $\mu g\ ml^{-1}$; streptomycin (Sm), 200 $\mu g\ ml^{-1}$; rifampicin (Rf), 100 $\mu g\ ml^{-1}$; cloramphenicol (Cm), 20 $\mu g\ ml^{-1}$; gentamycin (Gm) 20 or 50 $\mu g\ ml^{-1}$. Growth was routinely monitored spectrophotometrically at 600 nm.

DNA MANIPULATIONS AND CONSTRUCTIONS

Standard techniques were used for plasmid isolation, restriction enzyme digests, ligations, transformations, and agarose gel electrophoresis by Sambrook et al. (1989).

To construct pNP149 a 430 bp fragment internal to *fbaB* was amplified using genomic Rm1021 DNA as a template and the primers fbaB 5' ( 5'-ATATGGATCCTCCCGAATGAAACGATAACC-3') and fbaB3' (5'-TATAGGTACCGCGCTTCAGCACCTTGAT-3'). This band was cloned as a *Bam*HI / *Kpn*I fragment into pKNOCK-Gm as outlined by Alexyev (1999, Biotechniques 26:824-828), and transformed into competent *E. coli* S17-1 as described by Simon et al. (1983, Bio/Techniques 1: 784-791).

To construct pNP150 a 350 bp internal fragment to *tpiA2* was amplified using genomic Rm1021 DNA as a template and the primers tpiA2 5' (5'-ATATGATCCAGAACATGCACTGGGACGAT-3') and tpiA2 3' (TATAGGTACCGCTCGTAAGCCAGAAGAACG-3'). The band was gel purified and cloned as a *Bam*HI/*Kpn*I fragment into pBlueScriptII SK yielding pNP151. This band was subsequently isolated from pNP151 and ligated into pKNOCK-Gm outlined by Alexyev (1999) and transformed into competent *E. coli* S17-1 as described by Simon et al. (1983) yielding pNP150.

GENETIC TECHNIQUES

Mutagenesis of Rm1021 or RmG212 with Tn5 or Tn5B20 was carried out following the method of Finan et al. (1985, Cell 40: 869-877). Transductions, using Ø M12, were performed as described by Glazebrook (1991, Methods of Enzymol. 204: 398-418). Conjugations were carried out as described by Finan et al. (1988, J, Bacteriol. 170: 3396-3403).

Construction of *tpiB* and *fbaB* was accomplished by conjugating either pNP149 or pNP150 into Rm1021 and Rm5000 respectively. Single crossovers were selected for by plating on either VMM agar containing Sm and Gm (for crosses into Rm1021) or onto LB containing Rf and Gm (for crosses into

Rm5000). A number of colonies were purified in each case and had the point of insertion verified by transductional linkage to surrounding markers.

CHARACTERIZATION OF MUTANTS

To identify the insertion point of individual Tn*5* inserts, a modification of an arbitrary PCR protocol was utilized following the methods described by Caetano-Annoles (1993, PCR Methods Appl. 3: 85-92) and Raffa et al. (2002, Molec. Microbiol. 45:1599-1611). Strains containing either Tn*5* inserts were purified and the genomic DNA was used as a template. A low stringency PCR amplification using the primers IS50(1) (5'-CACGATGAAGAGCAGAAG-3') and DGEN(1) (5'-GGCCACGCGTCGACTAGTCAGNNNNNNNNNNACGCC-3') was carried out. The products of this reaction were subsequently amplified with a higher stringency PCR amplification using the primers IS50(2) (5'-TAGGAGGTCACATGGAAGTCAGAT-3') and DGEN(2) (5'-GGCCACGCGTCGACTAGTAGTCAG-3'). The primers IS50(1) and IS50(2) are found within the IS50 elements of Tn5 such that the IS50(1) primer is nested between IS50(2) primer and the sequence flanking the transposon. The DGEN(2) primer was designed to be complementary to the end of DGEN(1). The products of the second PCR reaction were gel isolated and sequenced using the IS50(1) primer using the method of Oresnik et al. (1998, Molec. Plant-Microbe Interact. 11: 1175-1185). Sequencing reactions were done with dye terminators and detected with an ABI automated sequencer at the university of Calgary Core DNA facilities. Sequence data were trimmed of IS50 sequences and database searches were done with the BLASTX program as outlined by Altschul et al. (1997, Nucleic Acids Res. 25: 3389-3402) against the *S. meliloti* database (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime).

EXAMPLE 1

**Effects of carbon sources on growth of *S. meliloti tpi*⁻ mutants on solid media**

A *tpiA*⁻ mutant of *S. meliloti* Rm 1021 produced as described above, was isolated as a slow-growth phenotype on a defined medium with rhamnose as a

sole carbon source, and identified as SRmA185. To ensure that SRmA185 was not carrying any other unrelated mutations, the Tn5 was transduced from SRmA185 into the wild-type Rm1021 by selecting for the transduction of the neomycin marker associated with the Tn5. The transductants were screened for the cotransduction of the rhamnose phenotype. One of these transductants was purified and saved as SRmA327.

The site of insertion of the transposon in the genome was determined by using an arbitrary PCR protocol. Fig. 4 shows the results of a BLASTN analysis comparing DNA extracted from the SRmA327 strain, with the *S. meliloti* Rm1021 database. The query sequence is the DNA immediately flanking the Tn*5* insert from the tpiA mutant, while the subject sequence shows the corresponding matching DNA from the *S. meliloti* Rm1021 database. These data show that the transposon is inserted into a gene that has been annotated as a triose phosphate isomerase. Fig 5 is a visual representation of the DNA map showing that the flanking DNA corresponds to the insertion of a nucleotide sequence identified as Seq. ID No. 1, being at the beginning of the tpiA1 region. The intergenic region of 126 base pairs between *tpiA1* and the next gene, *Y1024* makes it unlikely that these genes are cotranscribed.

Since the genome of Rm1021 has two *tpi* genes annotated, we wished to demonstrate that *tpiA* was in fact the triose-phosphate isomerase that was utilized in the glycolysis and gluconeogenesis pathways. PCR primers were designed and a knockout mutant, designated *tpiB*, of the second triose-phosphate isomerase annotated in the Rm 1021 genome was generated as described above. Based on annotation of the genome, the mutants were tested for their ability to grow on media incorporating erythritol as the sole carbon source. The results (Table 3) demonstrate that these *tpiB* mutants are in fact unable to use erythritol as a sole carbon source thereby confirming that *tpiB* is used for erythritol catabolism, and not glycolysis or gluconeogenesis.

TABLE 3. Carbon phenotypes of *S. meliloti* strains carrying *tpi* mutations

| Strains | Relevant Genotype | Glucose[a] | Succinate | Glycerol | Rhamnose | Fucose | Erythritol |
|---|---|---|---|---|---|---|---|
| Rm1021 | wild-type | ++ | ++ | ++ | ++ | ++ | ++ |
| SRmA327 | *tpiA1* | ++ | ++ | - | +/-[b] | ++ | ++ |
| SRmA355 | *tpiB1* | ++ | ++ | ++ | ++ | ++ | - |

[a] Plates were scored after incubation for 4 days at 30°C. Carbon source concentrations were 15 mM in all cases. Symbols: ++, same as wild-type; +, slow ; +/-, very slow; -, no growth.

[b] SRmA327 showed a negative phenotype for the first 4 days followed by a delayed growth.

EXAMPLE 2

**Biochemical activity of *S. meliloti* tpi mutants**

Bacterial cells to be used for enzyme assays were grown overnight in LB broth and used to inoculate flasks containing 500 ml of either LB or defined media containing 15 mM succinate, glucose or erythritol as a sole carbon source. Cultures were grown overnight in a shaker (G25 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., New Brunswick N.J.) at 300 rpm. Cultures were routinely streaked onto LB agar plates when they were harvested to ensure purity of the cultures before their extracts were used for enzyme assays.

Cells were pelleted by spinning them in a centrifuge (4,000 x g, 10 minutes) and resuspended (2 ml/g cell wet weight) in an extraction buffer containing 100 mM Tris.HCl, 5 mM ß-mercaptoethanol, and 1 mM $MgCl_2$. Cells were disrupted by two passages through a French Pressure cell (16,000 psi). The extract was subsequently pelleted (10,000 x g, 15 minutes, 5°C) and the supernatant was used as the cell-free extract. The extract was further centrifuged in a microfuge for 15 minutes to reduce NADH oxidase activity for assays that measured the disappearance of NADH. Triose-phosphate isomerase activity was

assayed following a method based on Lynch et al. (1975, Can. J. Microbiol. 21: 1560-1572). Specific activities were expressed as nmol NADH oxidized per minute per milligram protein. Protein was measured as described by Bradford using a protein determination reagent (Sigma) and bovine serum albumin as a standard, following the method described by Bradford (1976, Anal. Biochem. 72: 248-254).

The results show that there are two sources of trios-phosphate isomerase activity in *S. meliloti* Rm1021. One is from tpiA1, which appears to be constitutively expressed, the other is from tpiB1, which is really part of the operon involved in erythritol catabolism and is inducible by erythritol. When SRmA327 is grown using either glucose or succinate as a sole carbon source, tpiB activity is not induced. This strongly suggests that tpiA is the glycolytic triose phosphate isomerase.

TABLE 4. Triose phopsphate isomerase activity of wild-type and mutants

| **Strains** | **Relevant Genotype** | **LB** | **Glc[a]** | **Suc[b]** | **Ery[c]** |
|---|---|---|---|---|---|
| Rm1021 | Wild-type | 517 | 502 | 756 | 820 |
| SRmA327 | *tpiA1* | 25 | 25 | 32 | 348 |
| SRmA355 | *tpiB1* | 1,872 | 806 | 592 | ND[d] |
| SRmA366 | *tpiA1*, *tpiB1* | 6 | 2 | 13 | ND |

Data represent an average of duplicate assays expressed as specific activity (nmol/min/mg protein). There was less than 10% difference between replicates.

[a] Grown in VMM with 15 mM glucose as sole carbon source.

[b] Grown in VMM with 15 mM succinate as sole carbon source.

[c] Grown in VMM with 15 mM erythritol as sole carbon source

[d] ND, not determined because cultures cannot grow using erythritol as a sole carbon source.

EXAMPLE 3

**Effects of erythritol and glycerol on *tpi⁻ S. meliloti* mutants**

Since *tpiB* mutants were able to grow on either glycerol or rhamnose but were annotated as being a triose phosphate isomerase, we were curious whether a *tpiB* enzyme activity could rescue a *tpiA⁻* mutant. Accordingly, *S. meliloti* Rm1021 mutants generated as described previously, and characterized as having either *tpiA*, *tpiB* or both mutations, were streaked onto defined solid media containing either low or high concentrations of erythritol, and also, onto solid media containing either low or high concentrations of erythritol plus 15 mM glycerol.

The results in Table 5 show that in media containing an inducing sugar on which a *tpiB* mutant cannot grow (erythritol), plus a non-inducing sugar on which a *tpiB* mutant can grow (glycerol), growth of the *tpiB⁻* mutant does not occur. This result indicates that the inducing sugar is converted to a phosphorylated intermediate which cannot be removed. Since the glycerol is present, the cell attempts to grow and thereby this becomes toxic to the cell. A similar result has been recently shown by Richardson et al 2004 (2004, J. Bacteriolo 186: 8433-8442) with *Rhizobium leguminosarum* mutants unable to use rhamnose as a sole carbon source. However at low levels of erythritol, SRmA237 can grow on with glycerol thereby demonstrating that a *tpiA⁻* mutation can be rescued by induction of *tpiB*.

TABLE 5. Growth on solid media containing erythritol and/or glycerol as carbon sources[a], of *S. meliloti* strains carrying either *tpiA*⁻ and/or *tpiB* mutations.

| Strains | Relevant Genotype | Glc | Glyc | Ery | Ery(0.4)[b] | Glyc + Ery(0.4) | Glyc + Ery(2.5)[c] |
|---|---|---|---|---|---|---|---|
| Rm1021 | Wild-type | ++ | ++ | ++ | - | ++ | ++ |
| SRmA327 | *tpiA1* | ++ | - | ++ | - | + | ++ |
| SRmA355 | *tpiB1* | ++ | ++ | - | - | + | - |
| SRmA366 | *tpiA1, tpiB1* | ++ | - | - | - | - | - |

Abbreviations are as follows: Glc - glucose; Glyc - glycerol; Ery - erythritol;

[a] 15 mM final concentration of each carbon source was added unless otherwise noted

[b] 0.4 mM erythritol.

[c] 2.5 mM erythritol

EXAMPLE 4

**Effects of a *tpi⁻ S. meliloti* mutant on dry matter accumulation in alfalfa**

Alfalfa seeds (*Medicago sativa* cv. Rangelander) were surface-sterilized by soaking immersion for 5 minutes in 90 % ethanol, followed by a 10-minute soaking immersion in 1% hypochlorite. The seeds were then washed with at least 10 volumes of sterile distilled water. The seeds were subsequently sown onto 1 % water agar plates and placed into a germination chamber. Germinating seeds were aseptically planted by hand into a sterile sand-vermiculite that had been presoaked with Jensen's plant nutrient solution prepared as disclosed by Glazebrook (1991).

Plants were inoculated with cultures of *S. meliloti tpiA*⁻ mutant SRmA185 or SRmA327 that had been grown overnight in LB and diluted 1/100 into 10 ml of sterile distilled water to a titre of approximately 1 x $10^6$ cfu/ml. The 10 ml was used to inoculate a pot containing 10 alfalfa seedlings.

Plants were harvested 28-35 days after inoculation and dry weights of the shoots cut at the cotyledon were used to assess symbiotic proficiency. Bacteria were also re-isolated from nodules and tested to confirm they were true to the phenotype inoculated.

Table 6. Dry matter accumulation by alfalfa inoculated with *S. meliloti* strain SRmA185 carrying a *tpiA* mutation

| **Strains** | Dry matter (mg/plant)[a] | Percent wild-type |
|---|---|---|
| Rm1021 | 34.9 ± 3.0 | 100 |
| SRmA185 | 42.9 ± 2.8 | 122.9 |
| Uninoculated | 6.2 ± 0.4 | 17.7 |

[a] dry weight data are the average ± standard error of three independent replicates. Each replicate contained between 8 and 10 plants.

Statistical analysis of these results suggests that the mean increase between the mutant and the wild-type is sound at the 92.5% confidence level. This same experiment was replicated in three completely independent trials. In each case the average dry weight accumulation of SRmA327 was between 115-122% of the Rm1021. Paired Students t-test analyses on the difference of the means between the wild-type and SRmA327 suggest that the ability of a tpiA mutant to accumulate more dry weight than the wild-type is sound at greater than a 95% confidence interval (Table 7).

Table 7. Replication of dry matter accumulation by alfalfa inoculated with *S. meliloti* strain SRm327carrying a *tpiA* mutation

| Trial[a] | Rm1021 | SRmA327 | Uninoculated | Percent wild-type[b] |
|---|---|---|---|---|
| 1 | 30.2 | 36.4 | 6.5 | 121 |
| 2 | 81.4 | 93.7 | 7.1 | 115 |
| 3 | 43.5 | 51.1 | 5.2 | 118 |

[a] Each trial consisted of between 3-6 independent replicates and was harvested between 28-34 days after inoculation.

[b] Calculated as the mean of SRmA185 or SRmA327 divided by the corresponding wild-type value and expressed as a percentage.

[c] Data are presented as the means (mg dry matter/plant).

While this invention has been described with respect to the preferred embodiments, it is to be understood that various alternations and modifications may be made that will be apparent to those skilled in this art, e.g., for affecting and/or impairing and/or inactivating functioning triose-phosphate isomerase enzyme activities in other strains of *Rhizobium sp.*, *Bradyrhizobium sp.*, *Sinorhizobium sp.*, *Mesorhizobium* sp. and *Azorhizobium sp.*, and therefore, the scope of the present invention is to be considered limited solely by the appended claims.

DEPOSIT OF MICROORGANISMS

Samples of strains SRmA185 and SRmA327 of *Sinorhizobium meliloti* as disclosed herein were deposited at the INTERNATIONAL DEPOSITORY AUTHORITY OF CANADA (IDAC) of 1015 Arlington Street, Winnipeg, Manitoba, Canada, R3E 3R2 (Telephone: 204-789-6030; Fax: 204-789-2018) for patent purposes under the terms of the Budapest Treaty. The deposits were both made on February 23, 2005, and the deposit receipt numbers are 230205-1 (SRmA185) and 230205-2 (SRmA327).

**International Depositary Authority of Canada**
National Microbiology Laboratory, Health Canada
1015 Arlington Street
Winnipeg, Manitoba Canada R3E 3R2
Tel: (204) 789-2070
Fax:(204) 789-2097

International Form IDAC/BP/4

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT**
(issued pursuant to Rule 7.1 of the *Budapest Treaty* Regulations)

ATTACH COPIES OF THE ORIGINAL DEPOSIT CONTRACT AND VIABILITY STATEMENT

*This International Depository Authority accepts the deposit of the microorganism specified below, which was received by it on* February 23, 2005

To (Name of Depositor): Ivan J. Oresenik

Address: Department of Microbiology, University of Manitoba, Winnipeg, MB

**Identification of Deposit**

Reference assigned by depositor: SrmA185

Accession Number assigned by this IDA: 230205-01

The deposit identified above was accompanied by:

☐ a scientific description (specify):

☐ a proposed taxonomic designation (specify):

Signature of person(s)authorized to represent IDAC:

Date: February, 23 2005

**International Depositary Authority of Canada**
National Microbiology Laboratory, Health Canada
1015 Arlington Street
Winnipeg, Manitoba Canada R3E 3R2

Tel: (204) 789-2070
Fax:(204) 789-2097

International Form IDAC/BP/4

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT**
(issued pursuant to Rule 7.1 of the *Budapest Treaty* Regulations)

ATTACH COPIES OF THE ORIGINAL DEPOSIT CONTRACT AND VIABILITY STATEMENT

*This International Depository Authority accepts the deposit of the microorganism specified below, which was received by it on* February 23, 2005

To (Name of Depositor): Ivan J. Oresenik

Address: Department of Microbiology, University of Manitoba, Winnipeg, MB

**Identification of Deposit**

Reference assigned by depositor: SrmA327

Accession Number assigned by this IDA: 230205-02

The deposit identified above was accompanied by:

☐ a scientific description (specify):

☐ a proposed taxonomic designation (specify):

Signature of person(s)authorized to represent IDAC:

Date: February, 23 2005

International Form IDAC/BP/9

**STATEMENT OF VIABILITY**
(Issued pursuant to Rule 10.2 of the *Budapest Treaty* Regulations)

**Party to Whom the Viability Statement is Issued**

Name: Kirby Eades Gale Baker

Address: Box 3432 Stn. D, Ottawa, ON K1P 6N9

**Depositor**

Name: Ivan J. Oresenik

Address: Department of Microbiology, University of Manitoba, Winnipeg, MB

**Identification of the Deposit**

Accession Number given by the International Depository Authority: 230205-01

Date of the original deposit (or most recent relevant date): February 23, 2005

**Viability Test**

Viability of the deposit identified above was tested on (most recent date): Feb. 25, 2005

On the date indicated above, the culture was:

☑ viable

☐ no longer viable

Conditions under which the Viability Test were performed (to be filled in if the information has been requested and the results of the test were negative):

Signature of person(s) authorized to represent IDAC

Date: February 25, 2005

International Form IDAC/BP/9

**STATEMENT OF VIABILITY**
(Issued pursuant to Rule 10.2 of the *Budapest Treaty* Regulations)

**Party to Whom the Viability Statement is Issued**

Name: Kirby Eades Gale Baker

Address: Box 3432 Stn. D, Ottawa, ON K1P 6N9

**Depositor**

Name: Ivan J. Oresenik

Address: Department of Microbiology, University of Manitoba, Winnipeg, MB

**Identification of the Deposit**

Accession Number given by the International Depository Authority: 230205-02

Date of the original deposit (or most recent relevant date): February 23, 2005

**Viability Test**

Viability of the deposit identified above was tested on (most recent date): Feb. 25, 2005

On the date indicated above, the culture was:

☑ viable

☐ no longer viable

Conditions under which the Viability Test were performed (to be filled in if the information has been requested and the results of the test were negative):

Signature of person(s) authorized to represent IDAC

Date: February 25, 2005

SEQUENCE LISTING FREE TEXT

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| SEQ ID NO: 6 | Primer IS50(1) |
| SEQ ID NO: 7 | Primer DGEN(1) |
| SEQ ID NO: 8 | Primer IS50(2) |
| SEQ ID NO: 9 | Primer DGEN(2) |

CLAIMS:

1. A method for increasing dry matter production by a selected nitrogen-fixing legume, the method comprising:

selecting a rhizobial bacterial culture capable of infecting and establishing an effective symbiotic nitrogen-fixing relationship with said legume;

manipulating said rhizobial bacterial culture to impair a functioning triose-phosphate isomerase enzyme activity therein said rhizobial bacterial culture; and

inoculating said legume with an effective amount of said manipulated rhizobial bacterial culture wherein said functioning triose-phosphate isomerase enzyme activity has been impaired.

2. A method according to claim 1, wherein said inoculated legume is cultured into a plant.

3. A method according to claim 1, wherein said functioning triose-phosphate isomerase activity is impaired by genetically manipulating said rhizobial bacterial culture.

4. A method according to claim 3, wherein said functioning triose-phosphate isomerase activity is impaired by inserting a nucleotide sequence into a portion of rhizobial bacterial DNA, said nucleotide sequence configured to interfere with the synthesis of a triose-phosphate isomerase enzyme.

5. A method according to claim 3, wherein said functioning triose-phosphate isomerase activity is impaired by inserting a nucleotide sequence into a portion of rhizobial bacterial DNA, said nucleotide sequence configured to interfere with the activation of a triose-phosphate isomerase enzyme.

6. A method according to claim 3, wherein said functioning triose-phosphate isomerase activity is impaired by inserting a nucleotide sequence into a portion of rhizobial bacterial DNA, said nucleotide sequence configured to interfere with the functional operation of a triose-phosphate isomerase enzyme.

7. A method according to claim 3, wherein said functioning triose-phosphate isomerase activity is impaired by deleting a nucleotide sequence from a portion of rhizobial bacterial DNA encoding the synthesis of a triose-phosphate isomerase enzyme.

8. A method according to claim 3, wherein said functioning triose-phosphate isomerase activity is impaired by deleting a nucleotide sequence from a portion of rhizobial bacterial DNA encoding control over the activation of a triose-phosphate isomerase enzyme.

9. A method according to claim 3, wherein said functioning triose-phosphate isomerase activity is impaired by deleting a nucleotide sequence from a portion of rhizobial bacterial DNA encoding control over the functional operation of a triose-phosphate isomerase enzyme.

10. A method according to claim 1, wherein said legume is a forage legume.

11. A method according to claim 10, wherein said forage legume is selected from a group comprising alfalfa, clover, trefoil, crown vetch and milkvetch.

12. A method according to claim 1, wherein said legume is a grain legume.

13. A method according to claim 12, wherein said grain legume is selected from a group comprising beans, soybeans, faba beans, peas, chickpeas, lentils and lupins.

14. A method according to claim 1, wherein said rhizobial bacterial culture comprises a strain selected from a group comprising *Rhizobium sp.*, *Bradyrhizobium sp.*, *Sinorhizobium sp.*, *Mesorhizobium sp.* and *Azorhizobium sp.*

15. A method according to claim 14 wherein said rhizobial bacterial culture is a strain selected from a group comprising *Sinorhizobium meliloti* strains.

16. A method according to claim 15 wherein said rhizobial bacterial culture is a strain selected from a group comprising *Sinorhizobium meliloti* strain SRmA185 and *Sinorhizobium meliloti* strain SRmA327.

17. A composition for inoculating a legume, said composition comprising an effective amount of a rhizobial bacterial culture selected for its ability to infect and establish a nitrogen-fixing symbiosis with said legume, said rhizobial bacterial culture provided with an impaired triose-phosphate isomerase activity.

18. The composition of claim 17, wherein said rhizobial bacterial culture is manipulated to impair a functioning triose-phosphate isomerase enzyme activity therein said rhizobial bacterial culture.

19. The composition of claim 18, wherein said functioning triose-phosphate isomerase activity is impaired by inserting a nucleotide sequence into a portion of rhizobial bacterial DNA, said nucleotide sequence configured to interfere with the synthesis of a triose-phosphate isomerase enzyme.

20. The composition of claim 18, wherein said functioning triose-phosphate isomerase activity is impaired by inserting a nucleotide sequence into a portion of rhizobial bacterial DNA, said nucleotide sequence configured to interfere with the activation of a triose-phosphate isomerase enzyme.

21. The composition of claim 18, wherein said functioning triose-phosphate isomerase activity is impaired by inserting a nucleotide sequence into a portion of rhizobial bacterial DNA, said nucleotide sequence configured to interfere with the functional operation of a triose-phosphate isomerase enzyme.

22. The composition of claim 18, wherein said functioning triose-phosphate isomerase activity is impaired by deleting a nucleotide sequence from a portion of rhizobial bacterial DNA encoding the synthesis of a triose-phosphate isomerase enzyme.

23. The composition of claim 18, wherein said functioning triose-phosphate isomerase activity is impaired by deleting a nucleotide sequence from a portion of rhizobial bacterial DNA encoding control over the activation of a triose-phosphate isomerase enzyme.

24. The composition of claim 18, wherein said functioning triose-phosphate isomerase activity is impaired by deleting a nucleotide sequence from a portion of rhizobial bacterial DNA encoding control over the functional operation of a triose-phosphate isomerase enzyme.

25. A composition according to claim 17, wherein said legume is a forage legume.

26. A composition according to claim 25, wherein said forage legume is selected from a group comprising alfalfa, clover, trefoil, crown vetch and milkvetch.

27. A composition according to claim 17, wherein said legume is a grain legume.

28. A composition according to claim 27, wherein said grain legume is selected from a group comprising beans, soybeans, faba beans, peas, chickpeas, lentils and lupins.

29. A composition according to claim 17, wherein said rhizobial bacterial culture comprises a strain selected from a group comprising *Rhizobium sp.*, *Bradyrhizobium sp.*, *Sinorhizobium sp.*, *Mesorhizobium sp.* and *Azorhizobium sp.*

30. A composition according to claim 29 wherein said rhizobial bacterial culture comprises a strain selected from a group comprising *Sinorhizobium meliloti* strains.

31. A composition according to claim 30, wherein said rhizobial bacterial culture is selected from a group comprising *Sinorhizobium meliloti* strain SRmA185 and *Sinorhizobium meliloti* strain SRmA327.

32. A biologically pure rhizobial bacterial culture, said culture provided with an impaired functioning triose-phosphate isomerase activity.

33. A biologically pure rhizobial bacterial culture according to claim 32, wherein said culture comprises a strain selected from a group comprising *Rhizobium sp.*, *Bradyrhizobium sp.*, *Sinorhizobium sp.*, *Mesorhizobium sp.* and *Azorhizobium sp.*

34. A biologically pure rhizobial bacterial culture according to claim 32, wherein said culture comprises a strain selected from a group comprising *Sinorhizobium meliloti* strains.

35. A biologically pure culture of *Sinorhizobium meliloti* strain SRmA185.

36. A biologically pure culture of *Sinorhizobium meliloti* strain SRmA327.

Fig. 1

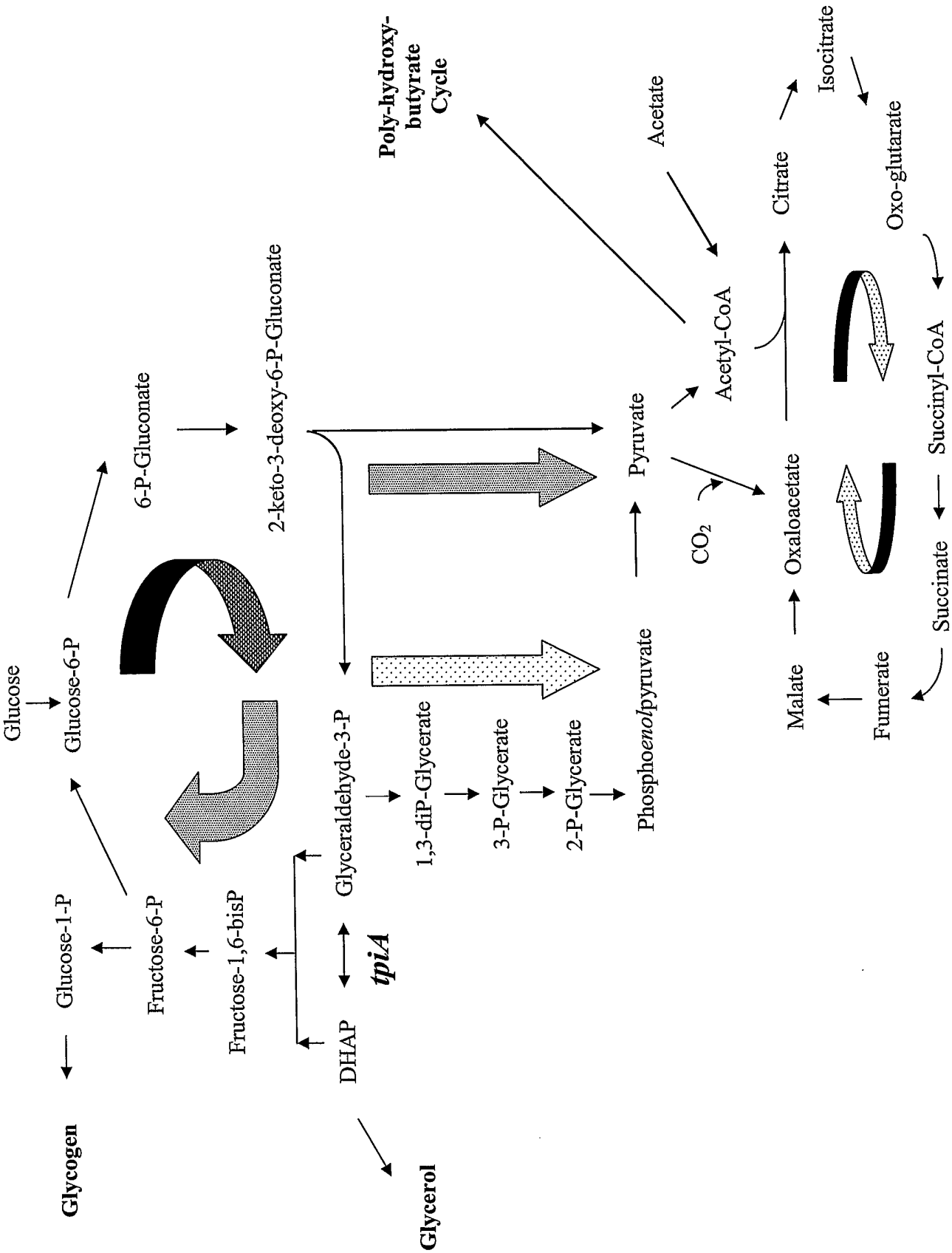
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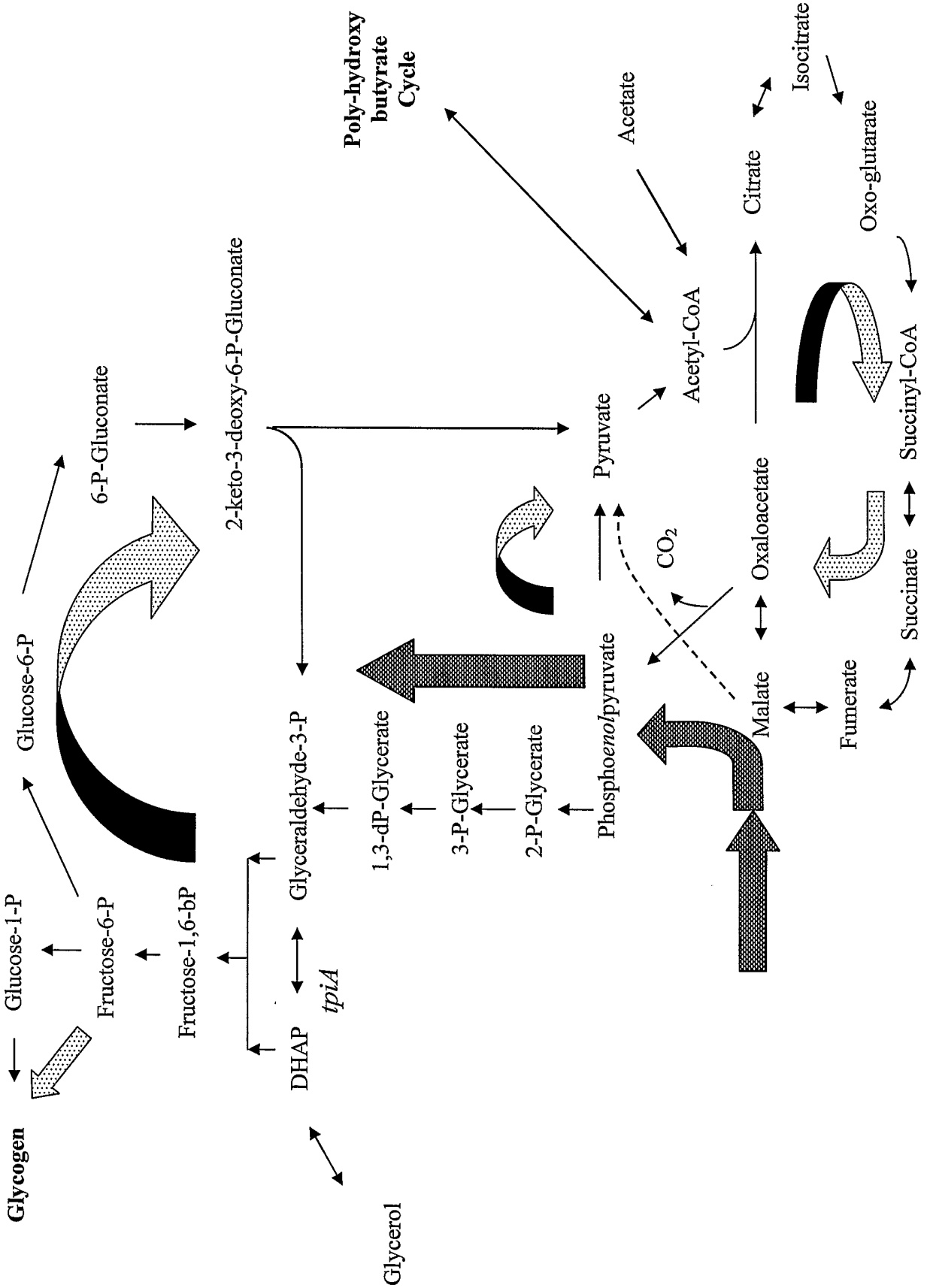
Fig. 2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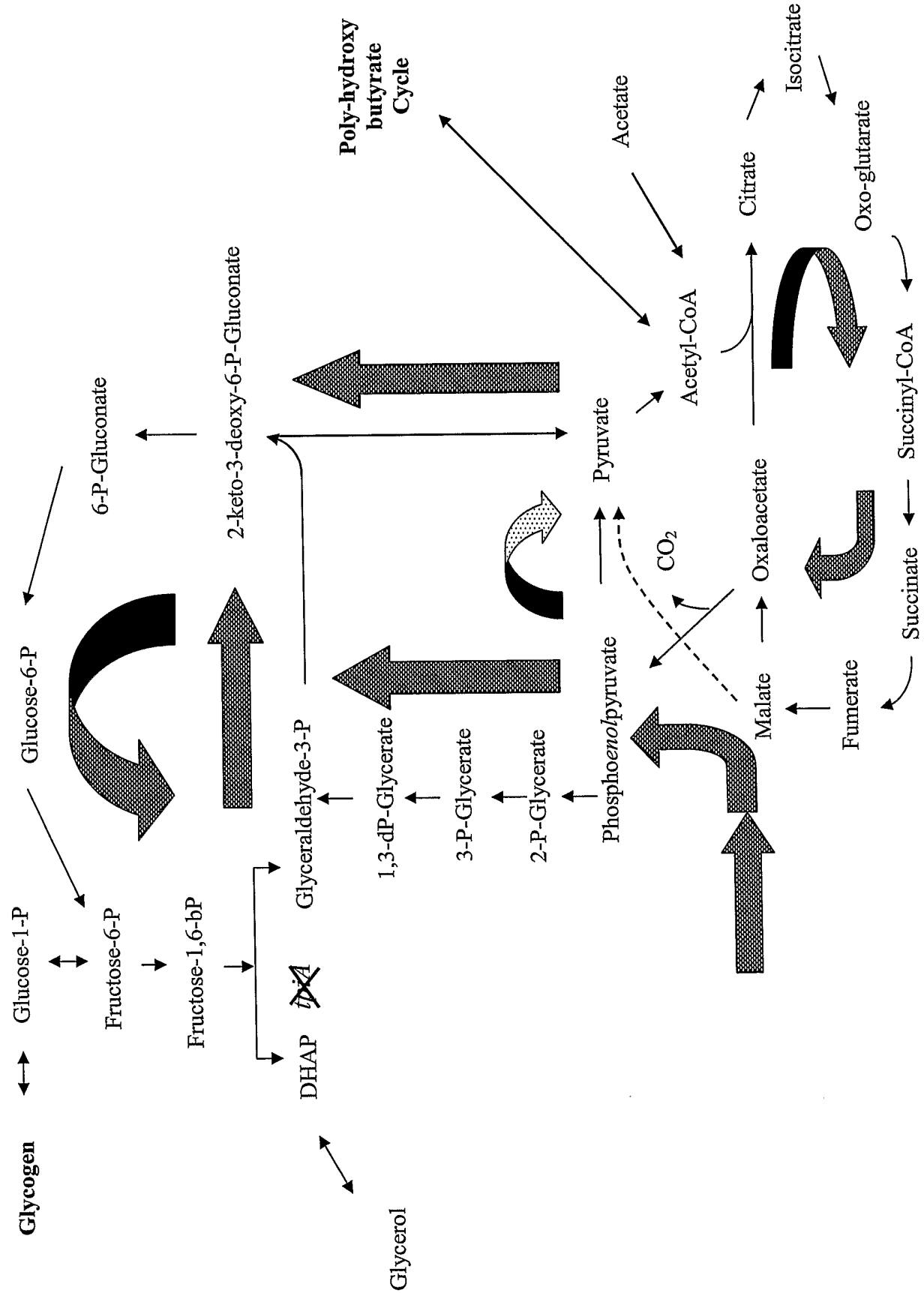
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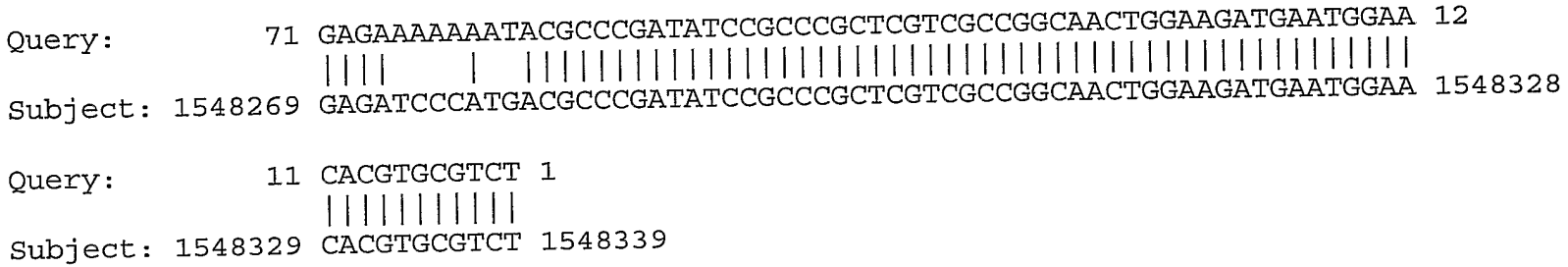
Fig. 4

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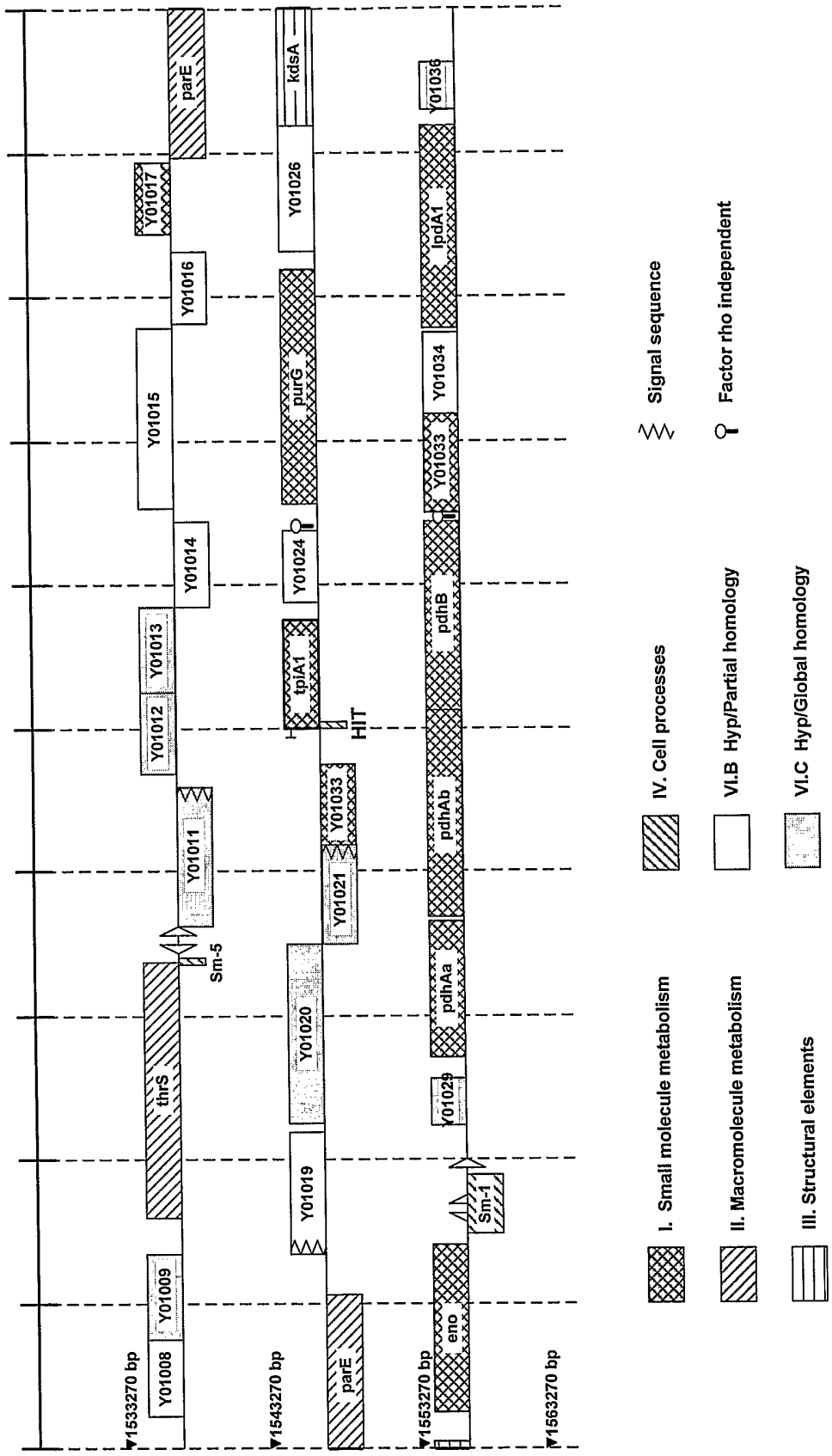
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