ORGANIC ACID-TOLERANT MICROORGANISMS AND USES THEREOF FOR PRODUCING ORGANIC ACIDS

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Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

Related U.S. Application Data

Provisional application No. 61/647,001, filed on May 15, 2012.

Int. Cl.
C12P 7/52  (2006.01)
C12P 7/40  (2006.01)
C12P 7/04  (2006.01)
C12N 9/88  (2006.01)
C12P 7/42  (2006.01)

U.S. Cl.
USPC 435/141; 435/132; 435/136

Field of Classification Search
None

See application file for complete search history.

References Cited

U.S. PATENT DOCUMENTS

8,048,624 B1  11/2011 Lynch

OTHER PUBLICATIONS

Gonzalez et al., Genetics and Molecular Features of Bacterial Dimethylsulfoxonopropionate (DMSP) and Dimethylsulfide (DMS) Transformations, in Handbook of Hydrocarbon and Lipid Microbiology, K.N. Timmis, Editor. 2010, Springer Berlin Heidelberg. p. 1201-1211.

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ABSTRACT

Organic acid-tolerant microorganisms and methods of using same. The organic acid-tolerant microorganisms comprise modifications that reduce or ablate AcasA activity or AcasA homolog activity. The modifications increase tolerance of the microorganisms to such organic acids as 3-hydroxypropionic acid (3HP), acrylic acid, and propionic acid. Further modifications to the microorganisms such as increasing expression of malonyl-CoA reductase and/or acetyl-CoA carboxylase provide or increase the ability of the microorganisms to produce 3HP. Methods of generating an organic acid with the modified microorganisms are provided. Methods of using acasA or homologs thereof as counter-selectable markers include replacing acasA or homologs thereof in cells with genes of interest and selecting for the cells comprising the genes of interest with amounts of organic acids effective to inhibit growth of cells harboring acasA or the homologs.

19 Claims, 8 Drawing Sheets
OTHER PUBLICATIONS


OPX nears commercial goal for bio-based acrylic acid. Feb 28, 2011:


Xu et al., Expression of Genes in Cyanobacteria: Adaptation of Endogenous Plasmids as Platforms for High-Level Gene Expression in &lt;i&gt;Synechococcus&lt;/i&gt; sp. PCC 7002, in Photosynthesis Research Protocols, R. Carpenter, Editor. 2011, Humana Press. p. 273-293.


FIG. 3

Graph showing the acrylate concentration over time for WT PCC 7002 and Abiotic control.
1. WT PCC 7002
2. Spontaneous mutant
3. ΔacsA
4. ΔacsA/pAQ1_acsAW49L
5. ΔacsA/pAQ1_acsA
ORGANIC ACID-TOLERANT MICROORGANISMS AND USES THEREOF FOR PRODUCING ORGANIC ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 USC §119(e) to U.S. Provisional Patent Application 61/647,001 filed May 15, 2012, the entirety of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under FA9550-11-1-0038 awarded by the USAF/AFOSR and DE-FC02-07ER64494 awarded by the US Department of Energy. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to organic acid-tolerant microorganisms and uses thereof for producing organic acids.

BACKGROUND

Production of industrially useful chemicals has conventionally focused on the use of petroleum-like compounds as starting materials. However, various factors have increased interest in the production of such chemicals through microorganism-mediated bioconversion of biomass and other renewable resources.

Accordingly, the U.S. Department of Energy (DOE) recently identified several “building block” chemicals to be produced via microorganism consumption of biomass. The identified chemicals include 1,4 sucrose, fumaric and malic acids, 2,5 furan dicarboxylic acid, 3-hydroxypropionic acid (3HP), aspartic acid, glutamic acid, tauric acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/ribitol. These chemicals can be converted to high-value, bio-based chemicals or materials.

As an example, 3HP can be readily transformed into a variety of commodity chemicals such as acrylic acid, methyl acrylate, and 1,3-Propanediol. These commodity chemicals represent a multi-billion dollar a year industry and are used in the production of plastics, coatings, and fibers. U.S. demand for acrylic acid in particular is growing, exceeding 1x10^6 kg/year. The current means of synthesizing acrylic acid include oxidation of propylene. A thermodynamically favorable pathway for microbial production of acrylic acid has not been identified.

One hurdle facing the microbial production of industrially useful chemicals is that many, including 3HP, are toxic to the microbes capable of producing them. Recently, efforts have been made not only to increase microbial output of the chemicals but also to increase microbial tolerance to the chemicals. Some of these efforts have focused on the production of 3HP in the heterotrophic microbe Escherichia coli. See, e.g., U.S. Pat. No. 8,048,624 to Lynch, U.S. Pat. 2011/0125118 to Lynch, U.S. Pat. 2010/0210017 to Gill et al., and Warnecke et al. Metabolic Engineering (2010) 12:241-250.

While focusing on chemical production in heterotrophic microorganisms is a valuable strategy, a potential problem is the availability of carbon and energy sources such as food-based commodities and/or sugars derived from lignocellulosic biomass. An attractive alternative is to use phototrophic microorganisms, such as cyanobacteria. These microorganisms can produce chemical products from CO2 and light energy without relying on consumption of higher-value carbon sources that can be used for other purposes, such as producing food, fuel, or other certain chemicals.

There is a need for microorganisms capable of producing high yields of industrially useful chemicals and having increased tolerance against those chemicals. There is also a need for microorganisms that use non-food-based feedstock in such production.

SUMMARY OF THE INVENTION

The present invention addresses the aforementioned needs by providing microorganisms with increased tolerance to organic acids. The present invention also provides microorganisms genetically modified to produce organic acids. Methods of producing organic acids with the microorganisms described herein are also provided.

Some versions of the invention provide an organic acid-tolerant microorganism comprising a modification that reduces or ablates AcsA activity or AcsA homolog activity in the microorganism, wherein tolerance to an organic acid selected from the group consisting of 3-hydroxypropionic acid (3HP), acrylic acid, and propionic acid is increased compared to a corresponding microorganism not comprising the modification.

The modification is preferably a genetic modification. The genetic modification is preferably a genetic modification other than or in addition to one resulting in a W49L substitution in AcsA or a corresponding substitution in an AcsA homolog.

The microorganism is preferably a bacterium, more preferably a cyanobacterium, and most preferably a cyanobacterium selected from the group consisting of Synechococcus sp., Prochlorococcus sp., Synechocystis sp., and Nostoc sp.

The tolerance to the organic acid is preferably increased at least about 25-fold in the microorganism of the invention compared to the corresponding microorganism.

The tolerance to the organic acid may include a minimum inhibitory concentration (MIC) of at least about 10 mM to acrylic acid, an MIC of at least about 100 mM to 3HP, and an MIC of at least about 200 mM to propionic acid.

In preferred versions of the invention, the microorganism is capable of producing 3HP.

The microorganism may include at least one recombinant nucleic acid configured to overexpress a 3HP pathway enzyme. The at least one recombinant nucleic acid encoding the 3HP pathway enzyme may include a malonyl-CoA reductase gene, such as the malonyl-CoA reductase gene derived from Chloroflexus aurantiacus. The at least one recombinant nucleic acid encoding the 3HP pathway enzyme may additionally or alternatively include an acetyl-CoA carboxylase gene. The recombinant nucleic acids may be heterologous or may comprise heterologous elements.

Some versions of the invention provide a microbial culture comprising a microorganism as described herein and an amount of an organic acid. The amount of the organic acid may be selected from the group consisting of at least about 10 mM acrylic acid, at least about 100 mM 3HP, and at least about 200 mM propionic acid.

Further versions of the invention provide a method of producing an organic acid comprising culturing a microorganism as described herein in the presence of an amount of an organic acid. The organic acid may be selected from the group consisting of 3HP, acrylic acid, and propionic acid. The amount of the organic acid may be selected from the group consisting of...
of at least about 10 mM acrylic acid, at least about 100 mM 3HP, and at least about 200 mM propionic acid.

Another version of the invention includes methods of using acaA or homolog thereof as a counter-selectable marker. One method includes replacing an acaA or homolog thereof in a cell with a gene of interest and selecting for the cell comprising the gene of interest with an amount of an organic acid effective to inhibit growth of cells harboring a functional acaA gene or homolog thereof. The replacing preferably occurs through homologous recombination. The acaA or homolog thereof is preferably an acaA gene with at least one silent nucleic acid mutation that reduces background mutation frequency. The at least one silent nucleic acid mutation is preferably selected from the group consisting of T144C and G150C in acaA from Synechococcus sp. PCC 7002. The organic acid is preferably acrylate. The cell may comprise any cell in which acaA or a homolog thereof confers sensitivity to organic acids. Such a cell may include a Synechococcus sp. cell or a cell from any microorganism described herein, known in the art, or later discovered that harbors an acaA homolog. The selecting preferably results in the cell being homozygous for the gene of interest.

The objects and advantages of the invention will appear more fully from the following detailed description of the preferred embodiment of the invention made in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A depicts a schema for using acaA or a homolog thereof as a selection marker for introducing a DNA fragment of interest into the acaA or homolog chromosomal locus.

FIG. 1B depicts a schema for using acaA or a homolog thereof as a selection marker for introducing a DNA fragment of interest into a locus other than the acaA or homolog chromosomal locus.

FIG. 2A depicts growth of Synechococcus sp. PCC 7002 at OD730 as a function of time in the presence of 5 mM dimethylsulfoxonipropionate (DMSP).

FIG. 2B depicts growth of Synechococcus sp. PCC 7002 at OD730 as a function of time in the presence of 5 mM acrylate.

FIG. 2C depicts growth of a mutant pool of Synechococcus sp. PCC 7002 at OD730 as a function of time in the presence of 5 mM dimethylsulfoxonipropionate (DMSP) and 5 mM acrylate.

FIG. 3 depicts acrylate production from DMSP as a function of time for Synechococcus sp. PCC 7002 and an abiotic control.

FIG. 4A depicts growth of BSyn_006 (a acaA strain of Synechococcus sp. PCC 7002 having a barcode sequence in place of the acaA gene (PCC 7002 acaA:BC)) and pH as a function of time in CO2-limited conditions. Cultivation of BSyn_006 with 5 mM DMSP under CO2-limited conditions results in an increase in pH over time.

FIG. 4B depicts acrylate accumulation over time from cultivation of BSyn_006 with 5 mM DMSP and abiotic controls with 5 mM DMSP at pH 8.0, 8.25, and 8.5. The rate of DMSP degradation to acrylate acid increases with an increase in pH.

FIG. 5 depicts plating of wild-type Synechococcus sp. PCC 7002, a mutant generated from growth in the presence of acrylic acid, a acaA mutant, a acaA mutant comprising the pAQ1 plasmid containing acaA, and a acaA mutant comprising the pAQ1 plasmid containing acaA on media containing no organic acid, 5 mM acrylic acid, or 30 mM 3-hydroxypropionic acid (3HP).

FIG. 6 depicts relative acyl-CoA ligase activity of acaA for acetate, acrylate, propionate, and 3-hydroxypropionate (3HP).

FIG. 7 depicts two 3HP-production pathways, wherein 1 represents pyruvate kinase, 2 represents pyruvate dehydrogenase, 3 represents acetyl-CoA carboxylase, 4 represents malonyl-CoA reductase, 5 represents phosphoenolpyruvate carboxylase, 6 represents aspartate aminotransferase, 7 represents aspartate decarboxylase, and 8 represents β-alanine/α-ketoglutarate aminotransferase.

FIG. 8A depicts the percent of colonies positive for yellow fluorescent protein (YFP) or a barcode sequence resulting from use of acaA as a counter selection marker upon introducing the YFP or the barcode sequence into the chromosomal acaA locus of Synechococcus sp. PCC 7002.

FIG. 8B depicts levels of YFP expression from cells in which YFP was introduced into the glpK chromosomal locus using acaA as a counter selection marker and cells in which YFP was introduced into the acaA chromosomal locus using acaA as a counter selection marker.

DETAILED DESCRIPTION OF THE INVENTION

One version of the invention includes a microorganism wherein an acaA gene product or homolog thereof is functionally deleted. The acaA gene product (acaA) and homologs thereof are acetyl-CoA synthetases classified under Enzyme Commission (EC) number 6.2.1.11. Other names for these acetyl-CoA synthetases include “acetate-CoA ligases,” “acyl-CoA ligases,” and “acyl-activating enzymes.”

“Functional deletion” or its grammatical equivalents refers to any modification to a microorganism that ablates, reduces, inhibits, or otherwise disrupts production of a gene product, renders the gene product non-functional, or otherwise reduces or ablates the gene product’s activity. “Gene product” refers to a protein or polypeptide encoded and produced by a particular gene. In some versions of the invention, “functionally deleted acaA gene product or homolog thereof” means that the acaA gene is mutated to an extent that an acaA gene product or homolog thereof is not produced at all.

One of ordinary skill in the art will appreciate that there are many well-known ways to functionally delete a gene product. For example, functional deletion can be accomplished by introducing one or more genetic modifications. As used herein, “genetic modifications” refer to any differences in the nucleic acid composition of a cell, whether in the cell’s native chromosome or in endogenous or exogenous non-chromosomal plasmids harbored within the cell. Examples of genetic modifications that may result in a functionally deleted gene product include but are not limited to mutations, partial or complete deletions, insertions, or other variations to a coding sequence or a sequence controlling the transcription or translation of a coding sequence; placing a coding sequence under the control of a less active promoter; and expressing ribozymes or antisense sequences that target the mRNA of the gene of interest, etc. In some versions, a gene or coding sequence can be replaced with a selection marker or screenable marker. Various methods for introducing the genetic modifications described above are well known in the art and include homologous recombination, among other mechanisms. See, e.g., Green et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press (2012) and Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press (2001). Various other genetic modifications that functionally delete a gene product are described in the examples below.
Functional deletion can also be accomplished by inhibiting the activity of the gene product, for example, by chemically inhibiting a gene product with a small molecule inhibitor, by expressing a protein that interferes with the activity of the gene product, or by other means.

In certain versions of the invention, the functionally deleted gene product may have less than about 95%, less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 1%, or about 0% of the activity of the non-functionally deleted gene product.

In certain versions of the invention, a cell with a functionally deleted gene product may have about 95%, less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 1%, or about 0% of the activity of the gene product compared to a cell with the non-functionally deleted gene product.

In certain versions of the invention, the functionally deleted gene product may be expressed at an amount less than about 95%, less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 1%, or about 0% of the amount of the non-functionally deleted gene product.

In certain versions of the invention, the functionally deleted gene product may result from a genetic modification in which at least one base, at least two bases, at least three bases, at least four bases, at least five bases, at least ten bases, at least twenty bases, at least thirty bases, at least forty bases, or more, nonsynonymous substitutions are present in the gene or coding sequence of the gene product.

In certain versions of the invention, the functionally deleted gene product may result from a genetic modification in which at least one base, at least two bases, at least three bases, at least four bases, at least five bases, at least ten bases, at least twenty bases, at least thirty bases, at least forty bases, or more, bases are inserted in the gene or coding sequence of the gene product.

In certain versions of the invention, the functionally deleted gene product may result from a genetic modification in which at least about 1%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or about 100% of the gene product’s gene or coding sequence is deleted or mutated.

In certain versions of the invention, the functionally deleted gene product may result from a genetic modification in which at least about 1%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, or at least about 95%, or about 100% of a promoter driving expression of the gene product is deleted or mutated.

In certain versions of the invention, the functionally deleted gene product may result from a genetic modification in which at least about 1%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or about 100% of an enhancer controlling transcription of the gene product’s gene is deleted or mutated.

In certain versions of the invention, the functionally deleted gene product may result from a genetic modification in which at least about 1%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or about 100% of a sequence controlling translation of gene product’s mRNA is deleted or mutated.

In certain versions of the invention, the decreased activity or expression of the functionally deleted gene product is determined with respect to the activity or expression of the gene product in its unaltered state as found in nature. In certain versions of the invention, the decreased activity or expression of the functionally deleted gene product is determined with respect to the activity or expression of the gene product in its form in a corresponding microorganism. In certain versions, the genetic modifications giving rise to a functionally deleted gene product are determined with respect to the gene in its unaltered state as found in nature. In certain versions, the genetic modifications giving rise to a functionally deleted gene product are determined with respect to the gene in its form in a corresponding microorganism.

Some versions of the invention include a plurality of microorganisms, wherein greater than about 1%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or more of the plurality of microorganisms comprise a functionally deleted acsA gene product or homolog thereof. In some versions, the plurality of microorganisms is a microbial culture.

Genetic modifications that can be introduced into the acsA gene or homolog thereof to functionally delete the acsA gene product or homologs thereof, such as generating acsA knockouts, are described in the examples below.

The acsA gene is an acetyl-CoA synthetase gene in the exemplary cyanobacterium Synechococcus sp. PCC 7002, the coding sequence of which can be found in GenBank under accession number NC__010475.1 and is as follows:

```plaintext
# Example of sequence data
agtcccgaac aasacatgga attacactt ccagggagcc
gcgggggg gtcgacaggg caaggggccc
acctcaca cgcggggc cgggagggc
ggagccagc gtaggggctt accagccct ctagggcgg
gcggagagtc acccgacaggg ctttgggggg gaagggcgg
aacgaagtc ggatggtcct gagaaatggg acaaggtgct
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The acsA coding sequence in the exemplary organism Synechococcus sp. PCC 7002 encodes a protein included in GenBank under accession number YP_001735082.1, having the following amino acid sequence:

(MSRQID NO: 2)

Homologs of acsA include genes or gene products encoded thereby that are homologous to the acsA gene or its product. Proteins and/or protein sequences are “homologous” when they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity (e.g., identity) over 50, 100, 150 or more residues (nucleotides or amino acids) is routinely used to establish homology (e.g., over the full length of the two sequences to be compared). Higher levels of sequence similarity (e.g., identity), e.g., 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% or more, can also be used to establish homology. Accordingly, homologs of the genes or gene products described herein include genes or gene products having at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity to the genes or gene products described herein. Methods for deter-
mining sequence similarity percentages (e.g., BLASTP and BLASTN using default parameters) are described herein and are generally available. The homologous proteins should demonstrate comparable activities and, if an enzyme, participate in the same or analogous pathways. “Orthologs” are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same or similar function in the course of evolution. As used herein “orthologs” are included in the term “homologs”.

For sequence comparison and homology determination, one sequence typically acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequent coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence based on the designated program parameters. A typical reference sequence of the invention is a nucleic acid or amino acid sequence corresponding to acesA or other genes or products described herein.


One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity for purposes of defining homologs is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, and a cutoff of 100, M=5, N=−4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the two nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. The above-described techniques are useful in identifying homologous sequences for use in the methods described herein.

The terms “identical” or “percent identity”, in the context of two or more nucleic acid or polypeptide sequences, refers to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described above (or other algorithms available to persons of skill) by visual inspection.

The phrase “substantially identical”, in the context of two nucleic acids or polypeptides refers to two or more sequences or subsequences that at least about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 98%, or about 99% or more nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. Such “substantially identical” sequences are typically considered to be “homologous”, without reference to actual ancestry. Preferably, the “substantial identity” exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably, the sequences are substantially identical over at least about 150 residues, at least about 250 residues, or over the full length of the two sequences to be compared.

Non-limiting examples of gene-product homologs of the acesA gene in various microorganisms include the acetylcoenzyme A synthetase from Fischerella sp. JSC-11 (Genbank Accession No. ZP_000542831), the acetyl-coenzyme A from Mooreana producta ATCC 29413 (Genbank Accession No. ZP_003386065), the acetyl-CoA from C. proteinus sp. 11122 (Genbank Accession No. ZP_0005827472), the unnamed protein product from Thermosyphlococcus elongatus BP-1 (Genbank Accession No. ZP_001679439), the acetyl-CoA ligase from Cylindrospermopsis raciborskii CS-505 (Genbank Accession No. ZP_001679439), the acetyl-CoA synthetase from Nostoc punctiforme PCC 73102 (Genbank Accession No. ZP_001679439), the acetyl-CoA ligase from Micrococcus chthonoplastes PCC 7420 (Genbank Accession No. ZP_001679439), the acetyl-coenzyme A synthetase from Nodularia spumigena CCY3414 (Genbank Accession No. ZP_001679439), the acetyl-CoA synthetase from Micrococcus chthonoplastes PCC 7420 (Genbank Accession No. ZP_001679439),
YP_001669396.1), the acetate-CoA ligase from ‘Nostoc azollae’ 0708 (Genbank Accession No. YP_003723268.1), the acetate gene from Microcystis aeruginosa PCC 7806 (Genbank Accession No. CAO8486.1), the acetate-CoA synthetase gene from Microcystis vaginatis FGP-2 (Genbank Accession No. ZP_08490634.1), the acetate-CoA ligase gene from Raphidiopsis brookii D9 (Genbank Accession No. ZP_06304063.1), the acetate gene product from Acaryochloris marina MBIC11017 (Genbank Accession No. YP_00157064.1), the acetate-CoA synthetase from Acaryochloris sp. CCM4510 (Genbank Accession No. ZP_09248274.1), the acetate-CoA synthetase from Oscillatoria sp. PCC 6506 (Genbank Accession No. ZP_07113076.1), the acetate-CoA synthetase from Cyanotheceeae sp. PCC 7425 (Genbank Accession No. YP_002484565.1), the Acetate-CoA ligase from Lyngbya sp. PCC 8106 (Genbank Accession No. ZP_01627397.1), the unnamed product from Trichodesmium erythraeum IMS101 (Genbank Accession No. YP_722064.1), the acetate-CoA synthetase from Arthrospira platensis strain Paracsa (Genbank Accession No. ZP_06338853.1), the acetate-CoA ligase from Arthrospira maxima CS-328 (Genbank Accession No. ZP_03274675.1), the acetate-CoA synthetase from Arthrospira sp. PCC 8005 (Genbank Accession No. ZP_09782650.1), the acetate-CoA ligase from Arthrospira maxima CS-328 (Genbank Accession No. EDD2097.1), the acetate-CoA synthetase from Arthrospira sp. PCC 8005 (Genbank Accession No. CCE18403.1), the unnamed protein product from Cyanothecae sp. PCC 8802 (Genbank Accession No. YP_003138301.1), the acetate-CoA ligase from Cyanotechae sp. PCC 8802 (Genbank Accession No. ACV01466.1), the acetate-CoA synthetase from Cyanotechae sp. PCC 8801 (Genbank Accession No. YP_00237363.1), the acetate-CoA synthetase from Cyanotechae sp. ATCC 51472 (Genbank Accession No. ZP_08974038.1), the unnamed protein product from Synecococcus elongatus PCC 6301 (Genbank Accession No. ZP_08974038.1), the acetate-CoA synthetase from Synecococcus sp. ATCC 51412 (Genbank Accession No. YP_001803432.1), the acetate-CoA synthetase from Cyanotechae sp. CCY0110 (Genbank Accession No. ZP_01730332.1), the AMP-dependent synthetase and ligase from Crocosphaera watsonii WH8101 (Genbank Accession No. ZP_00514814.1), the acetate-CoA ligase from Synecococcus sp. PCC 7335 (Genbank Accession No. ZP_05036109.1), the acetate-CoA synthetase from Synecococcus sp. WH 8102 (Genbank Accession No. NP_897106.1), the acetate-CoA ligase from Synecococcus sp. WH 7805 (Genbank Accession No. ZP_01123920.1), the acetate-CoA ligase from Synecococcus sp. WH 8109 (Genbank Accession No. ZP_05788236.1), the acetate-CoA synthetase from Prochlorococcus marinus strain MIT 9313 (Genbank Accession No. NP_894222.1), the acetate-CoA synthetase from Prochlorococcus marinus strain MIT 9303 (Genbank Accession No. YP_001071906.1), the acetate-CoA synthetase from Synecococcus sp. WH 7803 (Genbank Accession No. YP_001224763.1), the acetate-CoA synthetase from Synecococcus sp. RS9917 (Genbank Accession No. ZP_01080065.1), the acetate-CoA synthetase from Synecococcus sp. WH 8016 (Genbank Accession No. ZP_08955323.1), the acetate-CoA ligase from Synecococcus sp. CC9311 (Genbank Accession No. YP_730758.1), the acetate-CoA synthetase from Prochlorococcus marinus strain MIT 9211 (Genbank Accession No. YP_001550915.1), the acetate-CoA ligase from Synecococcus sp. CC9502 (Genbank Accession No. YP_377326.1), the acetate-CoA ligase from Synecococcus sp. BL107 (Genbank Accession No. ZP_01467683.1), the acetate-CoA synthetase from Synecococcus sp. RS9916 (Genbank Accession No. ZP_01471857.1), the acetate-CoA synthetase from Synecococcus sp. CC9605 (Genbank Accession No. YP_381449.1), the acetate-CoA synthetase from Synecococcus sp. CB0205 (Genbank Accession No. ZP_07971118.1), the acetate-CoA synthetase from Synecococcus sp. RCC307 (Genbank Accession No. YP_001227601.1), the acetate-CoA synthetase from Synecococcus sp. CB0101 (Genbank Accession No. ZP_07973216.1), the acetate-CoA ligase from Cyanobium sp. PCC 7001 (Genbank Accession No. ZP_05043915.1), the acetate-CoA ligase from Synecococcus sp. WH 5701 (Genbank Accession No. ZP_01085120.1), the acetate gene product from Prochlorococcus marinus subsp. marinus strain CCMP1375 (Genbank Accession No. NP_875433.1), the acetate-CoA synthetase from Prochlorococcus marinus subsp. marinus strain CCMP1407 (Genbank Accession No. YP_291252.1), the acetate-CoA synthetase from Gloeobacter violaceus PCC 7421 (Genbank Accession No. NP_923105.1), the acetate-CoA synthetase from cyanobacterium UCYN-A (Genbank Accession No. YP_00342182.1), the acetate-CoA synthetase from Prochlorococcus marinus subsp. marinus strain CCMP1986 (Genbank Accession No. NP_09573232.1), the acetate-CoA synthetase from Prochlorococcus marinus subsp. marinus strain MIT 9312 (Genbank Accession No. YP_397116.1), the acetate-CoA ligase from Meiothermus ruber DSM 1270 (Genbank Accession No. YP_003507084.1), the acetate-CoA synthetase from Prochlorococcus marinus strain MIT 9215 (Genbank Accession No. YP_001483902.1), the acs gene product from Prochlorococcus marinus strain AS9601 (Genbank Accession No. YP_001009068.1), the acetate-CoA synthetase from Prochlorococcus marinus strain MIT 9515 (Genbank Accession No. YP_001011000.1), the acetate-CoA ligase from Prochlorococcus marinus strain MIT 9202 (Genbank Accession No. ZP_05137406.1), the acetate-CoA synthetase from Marinithrixus hydrothermalis DSM 14884 (Genbank Accession No. YP_004368660.1), the acetate-CoA synthetase from Prochlorococcus marinus strain MIT 9301 (Genbank Accession No. YP_001090869.1), the unnamed protein product from Nostoc sp. PCC 7120 (Genbank Accession No. NP_488297.1), the acetate-CoA ligase from Truera radiowircitus DSM 17093 (Genbank Accession No. YP_003503955.1), the acetate-CoA ligase from Halangium ochraceum DSM 14365 (Genbank Accession No. YP_00326991.1), the acetate-CoA synthetase from Gemmatum obscuriglobus UQM 2246 (Genbank Accession No. ZP_02733777.1), the acetate-CoA synthetase from Isophphaera pallida ATCC 43644 (Genbank Accession No. YP_00417960.1), the acetate-CoA synthetase from Chlororherpeton thalassium ATCC 35110 (Genbank Accession No. YP_001995147.1), the acetate-CoA ligase from Plantomycyes maris DSM 8797 (Genbank Accession No. ZP_01856987.1), the acetate-CoA synthetase from Thermus thermophiles HB8 (Genbank Accession No. YP_144514.1), the acetate-CoA ligase from Planctomyces lunnsu DSM 3776 (Genbank Accession No. YP_003632128.1), the acetate-CoA synthetase from Thermus thermophiles HB1 (Genbank Accession No. YP_004855.1), the acetate-CoA synthetase from Oceanithermus profundus DSM 14977 (Genbank Accession No. YP_00405785.1), the acetate-CoA synthetase from
The organic acid-tolerant microorganism of the present invention may include any microorganism that harbors an acaA gene or homolog thereof or expresses an acaA gene product or homolog thereof that is capable of being functionally deleted to render the microorganism more tolerant of organic acids. The microorganism may be eukaryotic, such as yeast, or prokaryotic, such as bacteria or archaea. Among bacteria, gram-positive, gram-negative, and ungrouped bacteria are suitable. Phototrophs, lithotrophs, and organotrophs are also suitable. In preferred versions of the invention, the microorganism is a phototroph, such as a cyanobacterium. Preferred cyanobacteria include those selected from the group consisting of Synechococcus sp., Prochlorococcus sp., Synechocystis sp., and Nostoc sp., with particularly suitable examples of Synechococcus sp. including Synechococcus sp. PCC 7942, Synechocystis sp. PCC 6803, and Synechococcus sp. PCC 7002. A benefit of phototrophs is that they require only CO2 as a carbon source and are not dependent on food-based commodities or other types of biomass for which there is a growing high demand.

Functional deletion of the acaA gene product or homolog thereof in the microorganism results in increased tolerance of the microorganism to organic acids compared to a corresponding microorganism. As used herein, “corresponding microorganism” refers to a microorganism of the same species having the same or substantially same genetic and proteomic composition as a microorganism of the invention, with the exception of genetic and proteomic differences resulting from the modifications described herein for the microorganisms of the invention. Such tolerance is with respect to any organic acid present within the organism or its growth medium, particularly those that may be present in high abundance. Non-limiting examples of organic acids to which the microorganisms of the present invention have increased tolerance include acetic acid, acrylic acid, aspartic acid, benzoic acid, butyric acid, citric acid, formic acid, fumaric acid, furan dicarboxylic acid (2,5-furandicarboxylic acid), glutaric acid, glutamic acid, heptanoic acid, hexanoic acid, 3-hydroxypropionic acid (3HP), isophthalic acid, itaconic acid, lactic acid, levulinic acid, malic acid, malonic acid, oxalic acid, pentanoic acid, phosphoric acid, propionic acid, pyruvic acid, succinic acid (1,4 succinic acid), and terephthalic acid, among others. The examples show various aspects of increased tolerance to exemplary organic acids 3-hydroxypropionic acid (3HP), acryl acid, and propionic acid.

One aspect of the increased tolerance to organic acids is an increase in the minimal inhibitory concentration (MIC) of a particular organic acid compared to a corresponding microorganism. MIC is the lowest concentration of an agent that will inhibit growth of a microorganism. An MIC can be determined by titrating the agent in the growth medium of the microorganism. The lowest concentration of the agent in which the microorganism is no longer able to grow is the MIC. Methods of culturing microorganisms and of detecting their growth are well known in the art and are not discussed in detail herein. A relative increase in MIC indicates a higher tolerance to an agent and indicates that the microorganism can grow in the presence of a higher concentration of the agent. Conversely, a relative decrease in MIC indicates a lower tolerance to an agent and indicates that the microorganism can grow only in the presence of a lower concentration of the agent.

Functional deletion of the acaA gene product or homolog thereof in the microorganism confers an MIC of at least about 10 μM, 25 μM, 50 μM, 75 μM, 100 μM, 250 μM, 500 μM, 1 mM, 25 mM, 50 mM, 70 mM, 100 mM, 125 mM, or 150 mM to acetic acid; an MIC of at least about 10 μM, 15 μM, 20
mM, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM, 200 mM, 225 mM, 250 mM, 260 mM, 300 mM, 350 mM, or more to 3HP; and/or an MIC of at least about 250 μM, 500 μM, 1 mM, 50 mM, 100 mM, 200 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, or more to propionic acid. Such MICs occur in at least Synechococcus sp. cyanobacteria, such as Synechococcus sp. PCC 7002 and Synechococcus sp. PCC 7942, when assayed at a pH of about 8. Such MICs also occur in Synechocystis sp., such as Synechocystis sp. PCC 6803, when assayed at a pH of about 8. Such MICs also occur in any other microorganism described herein, such as Prochlorococcus sp., Nostoc sp., or others.

Another aspect of increased tolerance is increased growth rate in the presence of a certain concentration of an organic acid or an equal growth rate in the presence of an increased concentration of an organic acid compared to a corresponding microorganism.

In various aspects of the invention, functional deletion of the aceA gene product or homolog thereof in the microorganism confers at least about a 1.5-fold, a 5-fold, a 10-fold, a 15-fold, a 25-fold, a 50-fold, a 75-fold, a 100-fold, a 125-fold, a 150-fold, a 250-fold, a 1,000-fold, 1,250-fold, a 1,500-fold, a 1,750-fold, a 2,000-fold, a 2,250-fold, a 2,500-fold, a 2,750-fold, a 3,000-fold, a 3,250-fold, or a 3,500-fold increase in tolerance against an organic acid. The organic acid to which functional deletion of the aceA gene product confers such MICs may include acetic acid, 3HP, or propionic acid, among others. In some versions of the invention, for example, functional deletion of the aceA gene product in Synechococcus sp. PCC 7002 confers at least about a 2,800-fold increase in MIC for acetic acid, at least about a 26-fold increase in MIC for 3HP, and at least about a 100-fold increase in MIC for propionic acid at pH of about 8 (see examples below).

The increased tolerance to organic acids conferred by functional deletion of the aceA gene product or homolog thereof renders the microorganism particularly suited for producing high amounts of organic acids, many of which have industrial utility. Accordingly, the microorganism in some versions of the invention is capable of producing an organic acid that can be isolated for industrial purposes. The microorganism may be able to naturally make the organic acid, may be genetically modified to make the organic acid, or may be genetically modified to make increased amounts of the organic acid that it already makes. Non-limiting examples of organic acids that the microorganisms of the present invention can produce include acetic acid, aspartic acid, benzoic acid, citric acid, formic acid, fumaric acid, furen dicarboxylic acid (2,5-furandicarboxylic acid), gluconic acid, glutamic acid, 3-hydroxypropionic acid (3HP), isophthalic acid, itaconic acid, lactic acid, levocarboxylic acid, levulinic acid, malic acid, oxalic acid, phosphoric acid, propionic acid, pyruvic acid, succinic acid (1,4 succinic acid), and terephthalic acid, among others. In preferred versions of the invention, the microorganism is capable of making at least 3HP.

In preferred versions of the invention, the microorganism is genetically modified to enhance production of at least 3HP. This can be performed by increasing expression of a gene for any one or more of the enzymes catalyzing the various steps in a 3HP-production pathway. Non-limiting examples of suitable enzymes include pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase, malonyl-CoA reductase, phosphoenolpyruvate carboxykinase, aspartate aminotransferase, aspartate decarboxylase, and β-alanine/α-ketoglutarate aminotransferase. See FIG. 7. See also U.S. Pat. No. 8,048,624 to Lynch, U.S. Pub. 2011/0125118 to Lynch, U.S. Pub. 2010/0210017 to Gill et al., and Warnecke et al. Metabolic Engineering (2010) 12:241-250 for additional enzymes. Increasing expression can be performed using any of methods currently known in the art or discovered in the future. Examples include genetic modification of the microorganism as well as culturing the microorganism in the presence of factors that increase expression of the gene. Suitable methods for genetic modification include but are not limited to placing the gene under the control of a more active promoter, increasing the copy number of the gene, and/or introducing a translational enhancer on the gene (see, e.g., Olins et al. Journal of Biological Chemistry, 1989, 264(29):16973-16976). Increasing the copy number of the gene can be performed by introducing additional copies of the gene to the microorganism, i.e., by incorporating one or more exogenous copies of the native gene or a heterologous homolog thereof into the microbial genome, by introducing such copies to the microorganism on a plasmid or other vector, or by other means. “Exogenous” used in reference to a genetic element means the genetic element is introduced to a microorganism by genetic modification. “Heterologous” used in reference to a genetic element means that the genetic element is derived from a different species. A promoter that controls a particular gene is herein described as being “operationally connected” to the gene.

Accordingly, some microorganisms of the invention include at least one recombinant nucleic acid configured to overexpress a 3HP pathway enzyme. “Recombinant” as used herein with reference to a nucleic acid molecule or polypeptide is one that has a sequence that is not naturally occurring, has a sequence that is made by an artificial combination of two otherwise separated segments of sequence, or both. This artificial combination can be achieved, for example, by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acid molecules or polypeptides, such as genetic engineering techniques. “Recombinant” is also used to describe nucleic acid molecules that have been artificially modified but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated. A recombinant cell or microorganism is one that contains a recombinant nucleic acid molecule or polypeptide. “Overexpress” as used herein means that a particular gene product is produced at a higher level in one cell, such as a recombinant cell, than in a corresponding cell. For example, a microorganism that includes a recombinant nucleic acid configured to overexpress an enzyme produces the enzyme at a greater amount than a microorganism that does not include the recombinant nucleic acid.

In a preferred version of the invention, the microorganism is manipulated to express or increase expression of malonyl-CoA reductase. In some versions, the microorganism is modified to harbor a nucleic acid derived from Chloroflexus aurantiacus that encodes a malonyl-CoA reductase gene or a homolog thereof. The Chloroflexus aurantiacus malonyl-CoA reductase gene is included in GenBank under accession number AF530019 and has the following nucleotide sequence:

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GAGCGGGAAG CGAGCACTC CAGAAAAG TACCTAAT CTGCA
ACGCGTGG GCAGCAAGA TCGAAGCAG AGTCCGTC
GCTTCTCGC AGGGGACGG CAGCTTAC TTAGGGAC
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The *Chloroflexus aurantiacus* malonoy-CoA reductase gene product is included in GenBank under accession number AAS20429 and has the following amino acid sequence:

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MGTSGLKAG IALITGGMN IGSELTRFL ABGATVIISS
ERAKLTLA ERMQAGAOGP ARLIDLEVMD GSPDVAVRG
ISAIIVHQQ IDILVNGAS AGAQRRLAEI PILEAELGPG
AZETLI ASSAIKLHMLI REAPIMVGYG SAVVINTIP
SRAKRYGRP YVTPFASLNA LGQALKRLG ARGURNITP
PPEISDRIR TYFQMDLKL GRFIEDTHL PHTRRCLA
NDQGALERP PSVGQADLA VELAASSEA SGETEIVTH
GMEKPSCTE SLLARDTUL IDASRTGLL CADDIIEVRN
ALGMLALTCD SEVQRGPSA AALAQPQRAV QUERKLAGD
FTTPILPALP PRPADTAV PQAAGENTG IHAIVAILPA
THSHEPCVL EVVDLRMLD LATECITV IASELRAWQ
SQRILQGAA RQRPVIPILN GDQNMNVVQ RQASSAQQOL
IRVPHHEAEL DQCGASAGAD HLVPPWVQ IVRPAHSLR
GLEFACTTA QLHNQORHIN RITNIPANI SATTGARAS
VWAEELIGL HLKAINTAG QGASGQGIG RLLASHGAR
MLAARHKL KQMQADLSEQ LAYTVTTQV DRVHPAGCD
VSEAOALLDL VERTLAPAT VOYYINAGI AGVEEWMID
PVEIKHKPIL ALNISLYLM KKLAMPKQG GOQILNWS
YFGNQGDIY PAQRPHADD SQAQRAMAE VPARFGPRL
QINAPAPGV EGERLRCGE RGPLFARAR LILHEQKLM
LNAAILAAN TDESHNYEL ELLPGFVMAV LEQNPAPAA
LRELARRTPS EOKPAASSS ALLMRIAKL LLNHOYQG
VLAPDIFAH NLNPFFFFTR AQINDEARNQ RPQMNAXYL
QRMQTFPOA MAFYVMDR OSUGTFHPS CILYXERTT
GGGPSLPLSP ERLAEIVGST VYIENHIHL TNLARAYL
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Exemplary homologs of the *Chloroflexus aurantiacus* malonoy-CoA reductase gene product include but are not limited to the short-chain dehydrogenase/reductase SDR from *Chloroflexus aggregans* DSM 9485 (Genbank Accession No. YP_002462600.1), the short-chain dehydrogenase/reductase SDR from *Oscillochloris trichoides* DG6 (Genbank Accession No. ZP_07664596.1), the short-chain dehydrogenase/reductase SDR from *Roseiflexus castenholzii* DSM 13941 (Genbank Accession No. YP_001433009.1), and others. The genes encoding these gene products can be found in GenBank.

In some versions of the invention, the microorganism is manipulated to express or increase expression of acetyl-CoA carboxylase, either alone, with malonyl-CoA reductase, or with other enzymes. This can be performed by introducing exogenous acetyl-CoA carboxylase subunit genes into the microorganism, by introducing highly expressed promoters in front of the endogenous acetyl-CoA carboxylase subunit genes, by increasing translational efficiency, or by other means. In bacteria, acetyl-CoA carboxylase is a multisubunit enzyme that is encoded by four genes, accA, accB, accC, and accD. Exemplary acetyl-CoA carboxylase subunit genes for use in the present invention can be those found in *Synechococcus* sp. PCC 7002 or homologs thereof. The complete genome of *Synechococcus* sp. PCC 7002 can be found in GenBank under Accession No. NC_010475. The gene for accA can be found at positions 2536162-2537139 of NC_010475, the gene product of which has a sequence represented by GenBank Accession No. YP_001735676.1. The gene for accB can be found at positions 60707-61204 of NC_010475, the gene product of which has a sequence represented by GenBank Accession No. YP_001733325.1. The gene for accC can be found at positions 2210473-2211819 of NC_010475, the gene product of which has a sequence represented by GenBank Accession No. YP_001735364.1. The gene for accD can be found at positions 64484-65443 of NC_010475, the gene product of which has a sequence represented by GenBank Accession No. YP_001733331.1. Suitable promoters for increasing expression of these genes are known in the art. In some versions of the invention, an artificial operon comprising the accD, accA, accB, and accC subunits from *E. coli* can be introduced into the microorganism for expression or overexpression of acetyl-CoA carboxylase. See, e.g., US 2011/0165637 to Pfleger et al., which is incorporated herein by reference.

Other genetic modifications of the microorganism of the present invention include any of those described in U.S. Pat. No. 8,048,624 to Lynch, U.S. Pub. 2011/0125118 to Lynch, and U.S. Pub. 2010/0210017 to Gill et al., all of which are attached hereto. See also Warnecke et al., *Metabolic Engineering* (2010) 12:241-250. The genetic modifications in these references may be to enhance organic acid tolerance and/or increase organic acid production. The microorganism of the

Exogenous, heterologous nucleic acids encoding enzymes to be expressed in the microorganism are preferably co-optimized for the particular microorganism in which they are introduced. Codon optimization can be performed for any nucleic acid by a number of programs, including "GENECIPS"-brand expression optimization algorithm by DNA 2.0 (Menlo Park, Calif.), "GENEOPTIMIZER"-brand gene optimization software by Life Technologies (Grand Island, N.Y.), and "OPTIMUGENE"-brand gene design system by GenScript (Piscataway, N.J.). Other codon optimization programs or services are well known and commercially available.

In addition to the microorganism itself, other aspects of the present invention include methods of producing organic acids with the microorganisms of the present invention. The methods involve culturing the microorganism in conditions suitable for growth of the microorganism. The microorganism either directly produces the organic acid or acids of interest or produces organic-acid precursors from which the organic acid or acids of interest are spontaneously converted. Such conditions include providing suitable carbon sources for the particular microorganism along with suitable micronutrients. For eukaryotic microorganism and heterotrophic bacteria, suitable carbon sources include various carbohydrates. Such carbohydrates may include biomass or other suitable carbon sources known in the art. For phototrophic bacteria, suitable carbon sources include CO₂, which is provided together with light energy.

The microorganism of the present invention is capable of being cultured in high concentrations of the organic acid or acids that the organism is configured to produce. This enables increased production of the organic acid or acids of interest. The microorganism can be cultured in the presence of an organic acid in an amount up to the MIC for that organic acid. Various MICs for exemplary organic acids are described herein. Accordingly, the microorganisms of the invention (i.e., Synechococcus sp., Prochlorococcus sp., etc.) can be cultured in the presence of at least about 10 μM, 25 μM, 50 μM, 75 μM, 100 μM, 250 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 25 mM, 75 mM, 100 mM, 125 mM, or 150 mM acrylate acid; at least about 10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, or 350 mM 3HP; and/or at least about 250 μM, 500 μM, 750 μM, 1 mM, 25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, or 500 mM propionic acid. Such culturing preferably occurs at a pH of about 8.

Some versions of the invention include using acsA or a homolog thereof as a counter selection marker. The acsA or homolog thereof provides sensitivity to the organic acids acrylate acid, 3HP, and propionic acid. By replacing the native copy of acsA or homolog thereof with a gene of interest through double homologous recombination, one can select for cells which have gone through the recombination event by plating on acrylate acid or another organic acid as described herein. Acrylate acid is preferred because it has the lowest MIC value and requires the lowest concentration for selection. Through this method, one can introduce a gene or operon of interest onto a chromosome without the need for antibiotics. Additionally, one can plate on a higher organic acid concentration, i.e., one closer to the MIC value of the acsA mutant strain, to cure the strain of interest of any copies of the wild type chromosome. This is of particular interest because it can be difficult to create a homozygous strain using antibiotics as the selection agent.

One version comprises using acsA or homolog thereof as a counter selection marker for introducing DNA fragments of interest into the acsA or homolog locus. An exemplary version is shown in FIG. 1A. A host 10 is transformed with either linear DNA fragments or plasmid DNA comprising a sequence of interest 12 flanked by an upstream homologous sequence 14 and a downstream homologous sequence 16. For introducing the sequence of interest 12 into the acsA locus, the upstream homologous sequence 14 is preferably homologous to a region 15 3' of the acsA or homolog 19 on the host chromosome 18, and the downstream homologous sequence 16 is preferably homologous to a region 17 3' of the acsA or homolog 19 on the host chromosome 18. The homologous sequences 14, 16 are preferably at least about 25-base pairs (bp), about 50-bp, about 100-bp, about 200-bp, about 300-bp, about 400-bp, or about 500-bp long. The transformed culture is then plated in a concentration of an organic acid sufficient to select for transformed cells. In preferred versions, the transformed culture is plated in a sub-MIC concentration of an organic acid, such as a concentration greater than 0% the MIC but less than about 20% the MIC, about 40% the MIC, about 50% the MIC, about 60% the MIC, or about 70% the MIC. After colonies appear, the colonies are then plated on a higher concentration of the organic acid to ensure homozygosity.

Another version comprises using the acsA gene or homolog thereof as a counter selection marker to introduce DNA fragments of interest into loci other than an acsA or homolog locus without leaving an antibiotic resistance marker. An exemplary version is shown in FIG. 1B. The version shown in FIG. 1B is similar to that shown in FIG. 1A except that the acsA or homolog thereof 19 is not at the normal chromosomal locus. In the specific case of FIG. 1B, a homolog of acsA, acsA*, is included on a non-chromosomal plasmid 20. The acsA or homolog thereof 19 can also be at a locus on the chromosome 18 other than the native acsA or homolog locus. The upstream homologous sequence 14 in FIG. 1B is homologous to a region 15 3' of the acsA or homolog 19 on the non-chromosomal plasmid 20, and the downstream homologous sequence 16 is homologous to a region 17 3' of the acsA or homolog 19 on the non-chromosomal plasmid 20.

To increase the utility of acsA as a counter selection marker, two point mutations can be made, T144C and G150C. These point mutations maintain the same amino acid sequence but break up a run of base pairs that create a loss of function mutation hot spot. By creating these mutations, the background mutation frequency of this gene is reduced. This mutant version of acsA, acsA*, can be incorporated onto a non-chromosomal plasmid, such as the endogenous plasmid pAQ1 of a ΔacsA strain of PCC 7002. This base strain allows for incorporating a gene or operon of interest onto the pAQ1 plasmid without the use of antibiotics and quickly creating a homozygous strain.

The elements and method steps described herein can be used in any combination whether explicitly described or not.
The singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise.

Numerical ranges as used herein are intended to include every number and subset of numbers contained within that range, whether specifically disclosed or not. Further, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 2 to 8, from 3 to 7, from 5 to 6, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

All patents, patent publications, and peer-reviewed publications (i.e., "references") cited herein are expressly incorporated by reference to the same extent as if each individual reference were specifically and individually indicated as being incorporated by reference. In case of conflict between the present disclosure and the incorporated references, the present disclosure controls.

It is understood that the invention is not confined to the particular construction and arrangement of parts herein illustrated and described, but embraces such modified forms thereof as come within the scope of the following claims.

EXAMPLES

Summary of the Examples

One of the potential applications of metabolic engineering is the use of cyanobacteria to photosynthetically produce commodity chemicals traditionally derived from petroleum. In particular, acrylic acid has been identified as a high-value product that could be biologically derived. Unfortunately, a viable metabolic pathway has not previously been identified for its direct production.

As described in further detail below, a mutation resulting in increased tolerance to 3HP was discovered through investigating the metabolism of a sulfur compound, dimethylsulfoxoniopropionate (DMSP), by *Synechococcus* sp. PCC 7002 (PCC 7002). PCC 7002 was grown in the presence of DMSP to determine if it could be metabolized. This surprisingly resulted in the accumulation of acrylic acid, a by-product of DMSP metabolism, showing that *Synechococcus* sp. can produce acrylic acid. The accumulation of acrylic acid in the growth medium caused a stall in growth of the cyanobacteria, suggesting it had a toxic effect. After an additional incubation period, growth began to resume. It was originally hypothesized that the ability to grow in the presence of acrylic acid was the result of an adaptation to the stress through altered gene regulation. This hypothesis was later invalidated after an experiment was performed involving growing "unadapted" cells on solid medium containing acrylic acid. The number of colonies on the plate relative to a control suggested that a loss of function mutation was occurring that resulted in the ability to grow in the presence of acrylic acid. Additionally, all mutants obtained through growth in the presence of acrylic acid had increased tolerance levels to 3HP. The increase in tolerance caused by the mutation resulted in a strain of cyanobacteria constituting a platform for either 3HP or acrylic acid production.

Steps were taken to identify the site of the mutation. An RNA sequencing experiment was performed to characterize differential gene expression in the presence of either DMSP or acrylic acid. This data set was used to identify genes that had single base pair mutations relative to the wild type strain. Through this analysis, mutations were identified in the gene acsA. In order to determine if acsA was involved in acrylic acid and 3HP toxicity, a strain of PCC 7002 was created that had a deletion of the acsA gene. This strain, PCC 7002 ΔacsA, had increased MIC values compared to wild type PCC 7002. These experiments determined that it is a loss of function of acsA that results in increased tolerance. The gene acsA was annotated as an acetyl-CoA ligase.

In order to demonstrate the utility of the ΔacsA strain, a pathway for producing 3HP was introduced into both the wild type PCC 7002 and ΔacsA strains. Several pathways exist for the production of 3HP from central metabolites. The chosen pathway involves an enzyme from the CO₂ fixation pathway of the thermophilic bacterium *Chloroflexus aurantiacus*. In this pathway, malonyl-CoA is converted to 3HP through a two-step reaction catalyzed by the enzyme malonyl-CoA reductase. Results have shown that expression of malonyl-CoA reductase confers the ability to produce 3HP on the order of 50 μM.

The result of these experiments is an engineered strain of PCC 7002 that can produce 3HP and is more tolerant to 3HP than wild type PCC 7002 or other cyanobacterial species. Further work will increase the yield of 3HP. The approach to increasing yield will involve further metabolic engineering and optimizing of culturing conditions. To further engineer this strain, expression of the malonyl-CoA reductase will be optimized and genes related to making malonyl-CoA will be over-expressed. Additionally, the current and further engineered strains will be cultured in a photobioreactor in order to monitor 3HP production under optimal growth conditions, and culture parameters will be adjusted to increase yields. The outcome of this work will be a strain of cyanobacteria with optimized culturing conditions that will result in a competitive yield of 3HP.

Background and Significance of Examples

Engineering Bacteria to Produce Commodity Chemicals

A current focus of metabolic engineering and synthetic biology is the development of new methods for producing commodity chemicals that are traditionally produced from petroleum [1,2]. Demand for methods of bioconversion of renewable resources (biomass or CO₂) to these compounds has increased due to price volatility and reliance on foreign production of oil, concerns of increasing atmospheric CO₂, and increased consumer demand for "green" and sustainable products. An example of recent commercial success is the production of 1,3-propanediol (a precursor of nylon-like materials) by DuPont via *Escherichia coli* fermentation of corn sugar [3].

Another compound that could be produced from renewable sources is acrylic acid. Acrylic acid, traditionally produced through the oxidation of propene, is used in coatings, finishes, plastics, and superabsorbent polymers [4]. US demand for acrylic acid continues to grow, exceeding 1×10⁷ kg/year, and is outstripping current production [4]. For this reason, non-petroleum based, sustainable methods for producing acrylic acid would be of value. Unfortunately, a thermodynamically favorable pathway for complete biological production of acrylic acid has not been identified [5]. An alternative route would be biological production of 3-hydroxypropionic acid (3HP), followed by a non-biological catalytic conversion to acrylic acid. Additionally, 3HP can be converted to other commodity chemicals including acrylamide and 1,3-propanediol [6]. One company, OPX Biotechnologies, has developed a bio-based technology for producing acrylic acid, via *Escherichia coli* fermentation of sugars to 3HP [7].

Cyanobacteria as an Alternative to Heterotrophic Bacteria

One of the concerns of using heterotrophic bacteria and yeast for fuel and chemical production is the use of food based commodities as feedstock. As the global population continues to grow and the cost of agricultural commodities continua-
ues to rise, an alternative route for biological production of commodity chemicals may be needed. An attractive alternative is to use cyanobacteria to convert CO₂ and light energy directly into chemical products. Using CO₂ rather than organic carbon as an input circumvents the problem of using agricultural commodities and could potentially decrease costs. Species of cyanobacteria are susceptible to genetic modification and have well studied metabolisms [8,9]. Recently, cyanobacteria have been engineered to produce a variety of chemicals and fuels including ethanol, hydrogen, isobutyraldehyde, isoprene, sugars, and fatty acids [10-14].

In order for cyanobacteria to be effective host systems for chemical production, they will have to produce the compound of interest in high titers and have improved resistance to end product toxicity. As presented below, a mutant strain of cyanobacteria was isolated with dramatically increased tolerance to acrylic acid and 3HP. This mutation was identified through exploring the role cyanobacteria play in metabolism of the marine sulfur compound dimethylsulfoniodipropionate (DMSP).

Metabolism of the Sulfur Compound DMSP

DMSP is an organic sulfur compound produced by eukaryotic algae and plants that accounts for 1-10% of primary productivity in the oceans [16]. DMSP has been shown to act as an osmoprotectant, antioxidant, predator deterrent, and a sink for reduced sulfur in marine eukaryotic algae [17,18]. Upon its release into the water, DMSP is metabolized by bacterioplankton for use as a carbon and reduced sulfur source [19]. The catabolism of DMSP has the potential to supply 1-15% of total carbon demand and nearly all of the sulfur demand for these bacterial communities [20]. Additionally, cyanobacteria have been shown to account for 10-34% of total DMSP assimilation in light-exposed waters [21,22].

DMSP is broken down through two major pathways. These pathways involve either direct cleavage of DMSP into dimethylsulfide (DMS) and acrylic acid or an initial demethylation followed by a cleavage reaction to form methanethiol and acrylic acid [16, 23-25]. Methanethiol is then used as a reduced sulfur source in methionine biosynthesis, while acrylic acid can be further metabolized into 3HP and used as a carbon source [26,27]. Additionally, release of DMS into the atmosphere from marine waters has been identified as a key intermediate in the cycling of terrestrial and marine sulfur pools [28]. While several genes have been identified in DMSP metabolism, none have been found in cyanobacteria. Recent studies have shown that two different groups of cyanobacteria are involved in the metabolism of DMSP. These studies demonstrated that both Synechococcus and Prochlorococcus species are capable of assimilating radio labeled DMSP and methanethiol. In addition, four pure strains of Synechococcus were analyzed for DMSP assimilation. Two of the four strains were able to transport and assimilate DMSP, while another produced DMS [22]. Of the species of cyanobacteria currently being used in metabolic engineering, only one, Synechococcus sp. PCC 7002, is found in marine environments and potentially exposed to DMSP.

Example 1

Acrylic Acid is Produced from Incubation of DMSP with PCC 7002

Metabolism of DMSP can result in the accumulation of several metabolites, including acrylic acid and 3HP, and may alter growth patterns due to its use as a carbon and sulfur source. PCC 7002 was cultured in the presence of 5 mM DMSP and analyzed for the presence of acrylic acid and 3HP. Growth was determined by monitoring OD730 while metabolic byproducts were measured through high pressure liquid chromatography (HPLC) and gas chromatography (GC). During incubation with DMSP, an increase in OD730 similar to a control culture was observed for several doubling events, followed by a delay in increased OD730 (FIG. 2A). HPLC analysis determined that during the initial growth period acrylic acid was being produced, although not at a rate significantly beyond an abiotic control (FIG. 3). However, extended incubation of PCC 7002 with DMSP resulted in an increase in acrylic acid concentrations beyond the abiotic control (FIG. 3). PCC 7002 does not contain genes with homology to those known to be involved in DMSP metabolism, but DMSP has been previously shown to slowly degrade to dimethylsulfide and acrylic acid at an alkaline pH [48,49]. The data presented in FIGS. 2A & B support a hypothesis that DMSP breakdown is abiotic and is enhanced by the increased pH resulting from cultivation of PCC 7002 under CO₂ limitation. The cultures in this study were not agitated or supplemented with bubbled air, creating a CO₂ limited environment. When grown in the presence of 5 mM acrylic acid, PCC 7002 exhibited a long lag followed by growth at a rate equal to the control (FIG. 2B). Both delays in increasing OD730 were linked by the presence of acrylic acid, suggesting that acrylic acid was causing growth inhibition. The eventual increase in OD730 in both cultures was due to spontaneous mutants within the population which were able to grow without inhibition. Sub-culturing of the mutant pool derived from wild type (WT) PCC 7002 grown with DMSP into medium containing acrylic acid resulted in no delay in growth (FIG. 2C). From these experiments it was concluded that DMSP incubated in the presence of PCC 7002 results in the production of acrylic acid, acrylic acid concentrations less than 5 mM are inhibitory, and spontaneous mutants can arise that are not inhibited by this concentration of acrylic acid.

Example 2

Acrylic Acid and 3HP Cause Toxicity at Low Concentrations

Accumulation of organic acid anions in the cytoplasm of bacteria has been shown to block metabolic pathways and arrest growth [32,33]. In addition to blocking metabolic pathways, high concentrations of organic acids have been shown to reduce the proton motive force through dissociation across the membrane [34]. Because of this, the toxicity of organic acids generally increases with the hydrophobicity of the compound [35]. The minimum inhibitory concentration (MIC) for PCC 7002, Synechococcus sp. PCC 7942, and Synechocystis sp. PCC 6803 were determined for acrylic acid, 3HP, and propionic acid at a pH of about 8 (Table 1). In all three species, acrylic acid was significantly more toxic than propionic acid, which was more toxic than 3HP. Furthermore, the toxicity of acrylic acid (pKa 4.35) to PCC 7002 was shown to be pH dependent, with toxicity increasing with decreasing pH. The low MIC for acrylic acid explains why cultures grown with DMSP become growth inhibited. Cultures with DMSP only show growth inhibition when the accumulating acrylic acid concentration reaches inhibitory concentrations. This suggests that acrylic acid and not DMSP causes the inhibition of growth. The eventual increase in OD730 suggests that mutations can arise to overcome this inhibition.
TABLE 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Acrylic Acid</th>
<th>3HP</th>
<th>Propionic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechococcus sp.</td>
<td>75 µM</td>
<td>No Data</td>
<td>No Data</td>
</tr>
<tr>
<td>PCC 7942</td>
<td>75 µM</td>
<td>No Data</td>
<td>No Data</td>
</tr>
<tr>
<td>Synechocystis sp.</td>
<td>75 µM</td>
<td>No Data</td>
<td>No Data</td>
</tr>
<tr>
<td>PCC 7002 A*</td>
<td>75 µM</td>
<td>No Data</td>
<td>No Data</td>
</tr>
</tbody>
</table>

Example 3

A Mutation in an Acetyl-CoA Ligase Gene Increases Tolerance to Acrylic Acid and 3HP

When a dense culture of PCC 7002 was plated onto solid medium containing acrylic acid, colonies resulting from spontaneous mutants uninhibited by acrylic acid were observed. The mutation frequency when selecting for growth on 50 µM acrylic acid was 7 x 10^-6. When selecting for growth on 5 mM acrylic acid, the mutation frequency was 4 x 10^-6. The mutation frequency is the frequency that a mutant with a given phenotype is found within the population of a culture. For example a mutation frequency of 1 x 10^-6 suggests that in a population of 1 x 10^10 cells, there are 100 mutants. The observed mutation frequencies are suggestive of a loss of function mutation. All mutants obtained from medium containing 50 µM acrylic acid were able to grow on 5 mM acrylic acid. In addition, these colonies were able to grow in media containing concentrations of propionic acid and 3HP that were above the WT PCC 7002 MIC values. One of the mutants, PCC 7002 A*, was analyzed to determine to what degree the tolerance to organic acids had increased. MIC values for this strain are presented in Table 1. Tolerance to acrylic acid increased about 280-fold over WT PCC 7002 MIC values. Increased tolerance to 3HP and propionic acid was also observed (data not shown). Due to the increased tolerance to all three organic acids, the mutation may affect a gene that links the metabolism of acrylic acid, 3HP, and propionic acid.

In addition to looking at gene expression levels, the results from the RNA-sequencing experiment were used to identify mutations that resulted in increased tolerance to acrylic acid. An analysis for single nucleotide polymorphisms (SNP) on the data set for each condition was performed. In order to identify potential mutation candidates, two basic assumptions were made. First, growth in cultures containing DMSP and acrylic acid would require the same mutation. Second, the mutation is a base pair change, not a deletion or insertion. From the SNP analysis, mutations in five candidate genes were identified. One of these candidates was annotated as an acetyl-CoA ligase (acsA). The mutation resulted in the change of a highly conserved tryptophan residue to a leucine (W49L) in Synechococcus sp. PCC 7002. The mutation changes an FWGE amino acid sequence in Synechococcus sp. PCC 7002 to an FLGE amino acid sequence. This mutation was a result of a G146T substitution in the acsA coding sequence. The mutation was present in ~60% of reads that aligned to this segment of the open reading frame in both the DMSP and acrylic acid cultures. Manual inspection of control alignment data determined that this allele was only present in cultures containing DMSP and acrylic acid. The correlate of W49L is conserved in the acsA of Escherichia coli (GenBank NP_418493.1) and

Bacillus subtilis (GenBank NP_390846.1), among others, suggesting it is integral to a functional protein. See, e.g., Table 2.

TABLE 2

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Protein Sequence</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechococcus</td>
<td>acsA</td>
<td>F-W-G-E</td>
<td>Residues 48-51</td>
</tr>
<tr>
<td>PCC 7942</td>
<td></td>
<td></td>
<td>Residues 38-41</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>Acetyl-CoA ligase</td>
<td>F-W-G-E</td>
<td>Residues 38-41</td>
</tr>
<tr>
<td>P. fulva</td>
<td>Acetyl-CoA ligase</td>
<td>F-W-G-E</td>
<td>Residues 38-41</td>
</tr>
</tbody>
</table>

The W49L mutation residue resulted in an insoluble protein (data not shown) and, therefore, a non-functional protein. These data lead to the hypothesis that loss of function of acsA would result in the observed increase in organic acid tolerance.

Without being limited by mechanism, it was hypothesized that the AcsA acetyl-CoA ligase may have a substrate specificity that would allow it to add a coenzyme A (CoA) to all three organic acids, and that the CoA bound acids or downstream metabolism of these CoA bound acids caused toxicity. This hypothesis was tested by creating a knockout mutant of the acsA gene. This knockout was created by transforming wild type PCC 7002 with a DNA construct that would replace the acsA gene with an antibiotic resistance marker through homologous recombination. The resulting mutant, ΔacsA, was challenged with concentrations of acrylic acid, 3HP, and propionic acid above WT PCC 7002 MIC levels. In each case the ΔacsA mutant was able to grow without inhibition, including in the presence of >500 mM 3HP. Additionally, the ΔacsA mutant did not show any growth defects relative to wild type. These results show that loss of function of the acetyl-CoA ligase increases the tolerance of PCC 7002 to acrylic acid and 3HP.

To confirm this phenotype is the result of the deletion mutation, a complementation mutant was created by integrating a copy of acsA into a plasmid native to PCC 7002 ΔacsA. A corresponding mutant harboring a copy of acsA-W49L was also constructed. In the presence of acrylic acid, no strains harboring wild-type acsA were capable of growing while those harboring the mutant acsA were able to grow (FIG. 5).

In addition, the acsA gene was heterologously expressed in E. coli for protein purification and the substrate specificity was determined for AcsA in vitro (see below).

From these results, several conclusions can be drawn. DMSP is converted to acrylic acid by PCC 7002. Spontaneous mutations occur within the population that results in a drastically increased tolerance to acrylic acid, 3HP, and propionic acid. One mutation that can result in this phenotype is a loss of function or deletion of the acsA gene, which codes for an acetyl-CoA ligase.

Example 4

Deletion and Complementation Studies

Deletion and complementation studies were performed in various Synechococcus spp., and Synechocystis spp. The results are shown in Table 3. Replacement of the gene acsA in Synechococcus sp. PCC 7002 with an antibiotic resistance marker (aadA) resulted in a dramatic increase in tolerance to
acrylic acid, 3-hydroxypropionic acid (3HP), and propionic acid. An identical level of increase was observed when acsA was replaced with a 20 base-pair barcode sequence. This phenotype was complemented in an acsA deletion strain by expression of acsA under the native promoter in another locus on the chromosome (glpK). Complementation resulted in the restored sensitivity to both acrylic acid and 3HP. This phenotype was only partially complemented upon expression of acsAW49L from the glpK locus, showing that the AW49L mutation does not result in a complete loss of AcsA activity.

Homologous genes were identified in the cyanobacteria Synechocystis sp. PCC 6803 (sll0542; SEQ ID NOS:3 and 4) and Synechococcus sp. PCC 7942 (SYNPCC7942_1342; SEQ ID NOS:5 and 6). Replacement of the gene sll0542 in PCC 6803 with an antibiotic resistance marker resulted in an increase in tolerance to acrylic acid similar to the deletion of acsA in PCC 7002. When selecting for growth of Synechocystis sp. PCC 6803 on 50 μM acrylic acid, the mutation frequency was 2×10⁻⁶.

### TABLE 3

<table>
<thead>
<tr>
<th>Species</th>
<th>acrylic acid (mM)</th>
<th>3-HP (mM)</th>
<th>Propionic acid (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis sp. PCC 7942</td>
<td>0.003</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Synechocystis sp. PCC 6803</td>
<td>0.050</td>
<td>&gt;35</td>
<td>0.25</td>
</tr>
<tr>
<td>PCC 6803 sll0542::KmR</td>
<td>70</td>
<td>&lt;50</td>
<td>No Data</td>
</tr>
<tr>
<td>Synechococcus sp. PCC 7002</td>
<td>0.025</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>PCC 7002 acsA::aadA</td>
<td>70</td>
<td>260</td>
<td>&gt;400</td>
</tr>
<tr>
<td>PCC 7002 acsA::BC</td>
<td>70</td>
<td>250</td>
<td>No Data</td>
</tr>
<tr>
<td>PCC 7002 acsA::BC</td>
<td>0.015</td>
<td>15</td>
<td>No Data</td>
</tr>
<tr>
<td>glpK::acsA::aadA</td>
<td>7</td>
<td>No Data</td>
<td>No Data</td>
</tr>
<tr>
<td>PCC 7002 acsA::BC</td>
<td>7</td>
<td>No Data</td>
<td>No Data</td>
</tr>
<tr>
<td>glpK::acsA::AW49L::aadA</td>
<td>7</td>
<td>No Data</td>
<td>No Data</td>
</tr>
</tbody>
</table>

*BC, 20 base-pair barcode

### Example 5

**Substrate Specificity of AcsA**

The tolerance of PCC 7002 to acrylic acid and 3HP was dramatically increased by the deletion of the acetyl-CoA ligase gene (acsA). To obtain information regarding the AcsA-dependent toxicity, the substrate specificity of AcsA was determined.

Acetyl-CoA ligase purification: *Escherichia coli* BL21 containing plasmid pET28b with acsA were grown in 50 mL of LB to an OD₆₀₀ of 0.6 and induced with 1 mM IPTG. The induced culture was shaken at 37°C for 3 hrs. The culture was centrifuged and the resulting cell pellet was frozen at −20°C. The cell pellet was processed with Novagen BugBuster Protein Extraction Reagent (Part No. 70584-3). The resulting soluble protein fraction was used for His-tag purification using Ni-NTA agarose beads (Qiagen) and Pierce 0.8-mL centrifugation columns (Part No. 898689). Washes were done with 50 mM NaH₂PO₄, 300 mM NaCl, and 30 mM imidazole pH 8.0. The his-tagged protein was eluted with 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole pH 8.0. The insoluble fraction from the protein extraction was washed twice with BugBuster reagent followed by incubation with 400 µL 8M urea, 100 mM Tris-HCl, and 100 mM β-mercaptoethanol pH 8.2 for 30 min. The resulting solution was centrifuged at 16,000xg and the supernatant was collected. Protein fractions were run on a SDS-PAGE gel. His-tag purified protein fractions used in the acetyl-CoA ligase assay were concentrated and buffer exchanged using an Amicon Ultra-4 centrifugation column.

The buffer used for enzyme storage contained 0.1M NaH₂PO₄, 1mM EDTA, and 10% v/v glycerol.

Acetyl-CoA ligase activity assay: Acetyl-CoA ligase activity was determined by measuring the loss of free Coenzyme A (CoA) over time using Ellman’s reagent. (Riddles P W, Blakeley R L, & Zerner B (1979). Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid) — a reexamination. *Analytical Biochemistry* 94(1):75-81.) The enzyme reaction contained 10 mM ATP, 8 mM MgCl₂, 3 mM CoA, 0.1 M NaH₂PO₄, 1 mM EDTA, and 2 mM of the organic acid species. The concentration of AcsA in the reaction was 500 nM. Relative activity was determined by the amount of CoA consumed in 4 min relative to an acetate control. As shown in FIG. 6, AcsA has an activity towards acetate, acrylic acid, propionate, and 3HP.

**Example 6**

Use of acsA as a Counter-Selection Marker

The sensitivity of PCC 7002 to acrylic acid due to the activity of AcsA allows for one to directly integrate DNA fragments into the acsA locus and select for acrylic acid tolerance. This method results in integration into the PCC 7002 without the use of an antibiotic resistance marker. The use of antibiotic resistance markers is limited by the number of markers available and their tendency to result in heterozygous strains. PCC 7002 carries between 4-6 copies of the chromosome and the use of resistance markers can result in strains with a mixture of native and modified chromosomes. Use of AcsA as a counter-selection marker can quickly produce homozygous strains.

The acsA gene was used as a counter-selection marker to introduce DNA fragments of interest into the acsA loci on the chromosome, thereby deleting acsA without leaving an antibiotic resistance marker. The replacement of the chromosome was confirmed by DNA encoding yellow fluorescent protein (YFP), each flanked with 500 base-pair sequences homologous to regions directly 5' and 3' of acsA. The transformed culture was then plated on 50 µM acrylic acid. Colonies appeared after 3 days. The colonies were patched onto plates containing 50 µM acrylic acid and screened for the presence of the sequence of interest. Integration of the various sequences resulted in 30-50% of colonies being positive integrations. See FIG. 8A. Positive clones were streaked onto plates containing 10 mM acrylic acid. Colonies able to grow in the presence of 10 mM acrylic acid were homozygous for the integration. This method allows for fast and homozgyous chromosomal integrations.

The acsA gene was also used as a counter selection marker to introduce DNA fragments of interest into other loci on the chromosome without leaving an antibiotic resistance marker. In an acsA deletion strain of PCC 7002, acsA along with an antibiotic resistance marker was introduced onto the chromosome in the gene glpK. See, e.g., PCC 7002 acsA::BC glpK::acsA::aadA in Table 3. glpK was used as an insertion site because it is a pseudogene in PCC 7002 due to a frameshift mutation. The acsA resistance marker was then replaced with yellow fluorescent protein (YFP) under the expression of a constitutive promoter. This resulted in a strain of PCC 7002 with YFP integrated onto the chromosome without a residual marker. YFP expressed from the glpK locus was shown to have an equal level of expression to YFP expressed from the acsA locus. See FIG. 8B. These experiments demonstrate the one can directly select for integration into the acsA locus and...
use acsA as a counter selection tool to make clean integrations elsewhere on the chromosome.

Example 7

Using a Mutant Strain of PCC 7002 with Increased Tolerance, Introduce a Pathway for Producing 3HP and Apply Metabolic Engineering Principles to Increase Titers

While the ultimate goal is to produce acrylic acid through a single biological catalyst, no complete pathway has previously been demonstrated [5]. As an alternative, 3HP can be biologically derived and then catalytically converted to acrylic acid. A 3HP production pathway was introduced into PCC 7002 ΔacsA and its ability to produce 3HP from CO₂ and light energy was analyzed. Express a Malonyl-CoA Reductase in PCC 7002

FIG. 7 outlines two pathways for synthesizing 3HP from phosphoenolpyruvate (PEP). PEP is derivable in cyanobacteria through the oxidation of glyceraldehyde 3-phosphate, a product of CO₂ assimilation. While both pathways would result in a cofactor imbalance, the route via malonyl-CoA balances out the NADPH derived from the light reactions of photosynthesis and results in the net production of 2ATP and 2 NADH per 3HP. In order to introduce this pathway into PCC 7002, a malonyl-CoA reductase gene was heterologously expressed. Malonyl-CoA reductase from Chloroflexus aurantiacus was cloned into PCC 7002 ΔacsA [44]. C. aurantiacus is a phototrophic bacterium that produces 3HP as an intermediate in CO₂ fixation [45]. The malonyl-CoA reductase gene was introduced into a native plasmid under a highly expressed promoter [46]. Integration onto a native plasmid rather than the chromosome ensured a higher copy number of the gene. The native plasmid is required for growth, ensuring that the plasmid was not lost [46]. After integration was confirmed, the ability of the strain to produce 3HP was determined through HPLC. Preliminary results have shown that expressing malonyl-CoA reductase in wild-type PCC 7002 and PCC 7002 ΔacsA confers the ability to produce 3HP on the order of 50 μM. Further experiments will be performed to determine if the ΔacsA strain has an advantage with respect to yield and growth rate. We predict that the ΔacsA strain has an advantage with respect to yield and growth rate.

The Strain Will be Engineered to Increase Titers

Several strategies can be employed to increase 3HP production. These include altering the expression of the malonyl-CoA reductase by changing the promoter, introducing additional copies onto the plasmid, and/or codon-optimizing the gene. Codon optimization will ensure that rare codons exist in the coding sequence that would stall translation. Additionally, flux through this pathway can be increased by introducing highly expressed promoters in front of the acetyl-CoA carboxylase genes, thus increasing the pool of malonyl-CoA. Furthermore, a genome scale metabolic model can be used to predict genetic modifications that would provide additional flux through this pathway and correct cofactor imbalances [47]. These strategies will potentially increase titers of 3HP to be comparable with production systems using heterotrophic bacteria.

Conclusions from Examples

Increasing the tolerance of the cyanobacterium Synechococcus sp. PCC 7002 to the commodity chemicals acrylic acid and 3HP and increasing at least 3HP production through metabolic engineering make biological synthesis of these compounds from CO₂ a viable option.

REFERENCES

19. Yoch, D. C., Dimethylsulfoniopropionate: Its Sources, Role in the Marine Food Web, and Biological Degradation
<210> SEQ ID NO 1
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<212> TYPE: DNA
<213> ORGANISM: Synechococcus PCC7002

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Gln Tyr Gln Ala Leu Tyr Asp Arg Ala Lys Asn Asp Pro Glu Gly Phe 45  50
Trp Gly Glu Leu Ala Glu Glu Leu Glu Trp Phe Glu Lys Trp Asp 55  60
Lys Val Leu Asp Trp Gln Pro Pro Phe Ala Lys Trp Phe Val Asn Gly 65  70  75  80
Lys Ile Asn Ile Ser Tyr Asn Cys Leu Asp Arg His Leu Lys Thr Trp 85  90  95
Arg Lys Asn Lys Ala Ala Leu Ile Trp Gly Glu Pro Gly Asp Ser 100 105 110
Arg Thr Leu Thr Tyr Ala Glu Leu His His Glu Val Cys Gln Phe Ala 115 120 125
Asn Ala Met Lys Lys Leu Gly Val Lys Gly Asp Arg Val Gly Ile 130 135 140
Tyr Met Pro Met Ile Pro Glu Ala Val Val Ala Leu Ala Cys Ala 145 150 155 160
Arg Ile Gly Ala Pro His Thr Val Ile Phe Gly Gly Phe Ser Ala Glu 165 170 175
Ala Leu Arg Ser Arg Leu Glu Asp Ala Glu Ala Lys Leu Val Ile Thr 180 185 190
Ala Asp Gly Gly Phe Arg Lys Asp Lys Ala Val Pro Leu Lys Asp Gln 195 200 205
Val Asp Ala Ala Ile Ala Asp His His Ala Pro Ser Val Glu Asn Val 210 215 220
Leu Val Val Glu Arg Thr Lys Glu Pro Val His Met Glu Ala Gly Arg 225 230 235 240
Asp His Trp Trp His Asp Leu Glu Glu Val Ser Ala Asp Cys Pro 245 250 255
Ala Glu Pro Met Asp Ala Glu Asp Met Leu Phe Ile Leu Tyr Thr Ser 260 265 270
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Tyr Glu Gly Ala Pro Arg Pro Ser Asn Pro Gly Cys Tyr Trp Glu Ile 340 345 350
Ile Gln Lys Tyr Gly Val Thr Ile Phe Tyr Thr Ala Pro Thr Ala Ile 355 360 365
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<210> SEQ ID NO 3
<211> LENGTH: 1962
<212> TYPE: DNA
<213> ORGANISM: Synechocystis PCC6803

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Val Thr Glu Glu Ile Gly Ala Ile Ala Arg Pro Ala Glu Ile Arg Phe
595
Thr Asp Val Leu Pro Lys Thr Arg Ser Gly Lys Ile Met Arg Arg Leu
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Leu Arg Ser Leu Ala Ser Gly Gin Glu Ile Ser Gly Asp Thr Ser Thr
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Leu Glu Arg Thr Val Leu Asp Lys Leu Arg Glu Gly
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<210> SEQ ID NO 5
<211> LENGTH: 1971
<212> TYPE: DNA
<213> ORGANISM: Synechococcus PCC7942

<400> SEQUENCE: 5

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<210> SEQ ID NO 6
<211> LENGTH: 656
<213> ORGANISM: Synechococcus PCC7942
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Tyr Glu Ala Leu Cys Gln Lys Ala Ala Ala Asp Pro Val Ala Phe Trp
35  40  45
Gly Glu Leu Ala Ala Glu Leu Pro Trp Phe Glu Pro Trp Glu Gln
50  55  60
Thr Leu Asp Trp Ser Asn Pro Pro Phe Ala Iys Trp Phe Gly Gly Val
65  70  75  80
Lys Leu Asn Ile Ser His Asn Cys Leu Asp Arg Arg His Leu Thr Thr Trp
85  90  95
Arg Lys Asn Lys Ala Ala Ile Ile Trp Glu Gly Glu Pro Gly Asp Ser
100 105 110
Arg Thr Leu Thr Tyr Ala Gin Leu His Arg Glu Val Cys Gin Phe Ala
115 120 125
Asn Val Leu Lys Ser Leu Gly Ile Gin Lys Gly Asp Val Val Gly Val
130 135 140
Tyr Met Pro Met Ile Pro Gin Ala Ala Ile Ala Met Leu Ala Cys Ala
145 150 155 160
Arg Ile Gly Ala Val His Ser Val Val Phe Gly Gly Phe Ser Ala Glu
165 170 175
Ala Leu Arg Asp Arg Leu Val Asp Gly Gin Ala Lys Leu Val Val Thr
180 185 190
Ala Asp Gly Gly Trp Arg Lys Asp Ala Ile Val Pro Leu Lys Aep Ser
195 200 205
Val Asp Gin Ala Leu Glu Gly Asn Ala Cys Pro Ser Val Gin His Val
210 215 220
Leu Val Val Glu Arg Thr Lys Gin Asp Ile His Met Glu Pro Gly Arg
225 230 235 240
Asp His Trp Thr His Glu Leu Gin Thr Val Ser Ala Thr Cys Pro
245 250 255
Ala Glu Pro Met Asp Ser Gin Ala Asp Leu Leu Phe Val Leu Tyr Thr Ser
260 265 270
Gly Ser Thr Gly Lys Pro Lys Gly Val Val His Thr Thr Gly Gly Tyr
275 280 285
Asn Leu Tyr Ala His Ile Thr Gin Trp Thr Phe Asp Leu Gin Asp
290 295 300
Thr Asp Val Tyr Trp Cys Thr Ala Asp Val Gly Trp Ile Thr Gly His
305 310 315 320
Ser Tyr Ile Val Tyr Gly Pro Leu Ser Asn Gly Ala Thr Thr Leu Met
325 330 335
Tyr Glu Gly Ala Pro Arg Ala Ser Asn Pro Gly Cys Phe Thr Asp Val
Ile Glu Lys Tyr Gly Val Thr Thr Phe Tyr Thr Ala Pro Thr Ala Ile
340 345 350
Arg Ala Phe Ile Lys Met Gly Glu His Pro Ala Ala Arg Asp Leu
355
Ser Ser Leu Arg Leu Leu Gly Thr Val Gly Glu Pro Ile Asn Pro Glu
370 375 380
Ala Trp Ile Trp Tyr His Arg Val Ile Gly Gly Asp Arg Cys Pro Ile
385 390 395 400
Val Asp Thr Trp Trp Gly Thr Glu Thr Gly Gly His Met Ile Thr Ser
405 410 415
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420 425 430
Pro Gly Ile Leu Ala Asp Val Val Asp Leu Asp Gly Arg Ser Val Pro
435 440 445
Asp Asn Glu Gly Gly Tyr Leu Val Ile Arg His Pro Trp Pro Gly Met
450 455 460
Met Arg Thr Val Tyr Gly Asp Pro Asp Arg Phe Arg Arg Thr Tyr Trp
465 470 475 480
Glu His Ile Pro Pro Glu Asn Gly Glu Tyr Leu Tyr Phe Ala Gly Asp
485 490 495 500
Gly Ala Arg Arg Asp Ala Asp Gly Tyr Phe Trp Val Met Gly Arg Val
505 510
Asp Asp Val Ile Asn Val Ser Gly His Arg Leu Gly Thr Met Glu Ile
515 520 525
Glu Ser Ala Leu Val Ser His Pro Ala Val Ala Glu Ala Ala Val
530 535 540
Gly Arg Pro Asp Asp Leu Lys Gly Gly Ile Val Ala Phe Ile Thr
545 550 555 560
Leu Glu Ser Gly Ile Glu Thr Gly Asp Glu Leu Val Lys Asp Leu Lys
565 570 575
Lys His Val Ala Gln Glu Ile Ala Ile Ala Arg Pro Asp Glu Ile
580 585 590
Arg Phe Ser Glu Ala Leu Pro Lys Thr Arg Ser Gly Lys Ile Met Arg
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<210> SEQ ID NO 7
<211> LENGTH: 3898
<212> TYPE: DNA
<213> ORGANISM: Chloroflexus aurantiacus

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<br>**ID:** SEQ_108  **Length:** 1220  **Type:** DNA  **Organism:** Chloroflexus aurantiacus

**Sequence:**

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Gly Ala Thr Val Ile Ser Gly Arg Arg Ala Lys Leu Thr Ala
35  40  45

Leu Ala Glu Arg Met Glu Ala Glu Ala Gly Val Pro Ala Lys Arg Ile
50  55  60

Asp Leu Glu Val Met Asp Gly Ser Asp Pro Val Ala Val Arg Ala Gly
65  70  75  80

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<211> LENGTH: 645
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fulva

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65  70  75  80
Ile Asp Arg His Leu Ala Lys Arg Gly Asp Glu Val Ala Leu Ile Trp
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Glu Gly Asp Asp Pro Met Asp Ser Ala Arg Ile Thr Tyr Arg Glu Leu
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His Glu Glu Val Cys Arg Leu Ala Asn Val Leu Lys Ser Arg Gly Val
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Lys Lys Gly Asp Arg Val Cys Ile Tyr Met Pro Met Val Pro Glu Ala
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Ala Tyr Ala Met Leu Ala Cys Thr Arg Ile Gly Ala Val His Ser Val
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Val Phe Gly Gly Phe Ser Pro Asp Ala Leu Arg Asp Arg Ile Leu Asp
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Ala Asp Cys Arg Thr Val Ile Thr Ala Asp Glu Ala Val Arg Gly Gly
180 185 190
Lys Leu Ile Pro Leu Lys Ser Asn Val Asp Lys Ala Leu Ala Ser Cys
195 200 205
Pro Asn Val Ser Thr Val Leu Val Val Arg Thr Gly Asn Lys Val
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Asp Trp Asp Asp Lys Arg Asp Leu Trp Tyr Ala Glu Ala Val Glu Glu
225 230 235 240
Ala Gly Ala Asp Cys Pro Ala Glu Pro Met Asp Ala Glu Asp Pro Leu
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Phe Ile Leu Tyr Thr Ser Gly Ser Thr Gly Lys Pro Lys Gly Val Leu
260 265 270
His Ser Thr Ala Gly Tyr Leu Leu Gin Ala Ala Met Thr His Lys Tyr
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Val Phe Asp Tyr His Arg Gly Asp Ile Tyr Trp Cys Thr Ala Asp Val
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Gly Trp Val Thr Gly His Ser Tyr Ile Val Tyr Gly Pro Leu Ala Asn
305 310 315 320
Gly Ala Thr Ser Leu Ile Phe Glu Gly Val Pro Asn Tyr Pro Asp Thr
325 330 335
We claim:

1. An organic acid-tolerant microorganism comprising a modification that reduces or ablates AcsA activity or AcsA homolog activity in the microorganism, wherein tolerance to an organic acid selected from the group consisting of 3-hydroxypropionic acid (3HP), acrylic acid, and propionic acid is increased compared to a corresponding microorganism not comprising the modification, wherein the microorganism is a bacterium.

2. The microorganism of claim 1 wherein the modification is a genetic modification.

3. The microorganism of claim 1 wherein the modification is a genetic modification other than or in addition to one resulting in a W49I substitution in AcsA or a corresponding substitution in an AcsA homolog.

4. The microorganism of claim 1 wherein the microorganism is a cyanobacterium.

5. The microorganism of claim 1 wherein the microorganism is a cyanobacterium selected from the group consisting of Synechococcus sp., Prochlorococcus sp., Synechocystis sp., and Nostoc sp.

6. The microorganism of claim 1 wherein the tolerance to the organic acid is increased at least about 25-fold compared to the corresponding microorganism.

7. The microorganism of claim 1 wherein the microorganism is Synechococcus sp. and wherein the tolerance to the organic acid is selected from the group consisting of a minimum inhibitory concentration (MIC) of at least about 10 mM to acrylic acid, an MIC of at least about 100 mM to 3HP, and an MIC of at least about 200 mM to propionic acid.
8. The microorganism of claim 1 wherein the microorganism is capable of producing 3HP.

9. The microorganism of claim 1 wherein the microorganism includes at least one recombinant nucleic acid configured to overexpress a 3HP pathway enzyme.

10. A microbial culture comprising the microorganism of claim 1 and an amount of an organic acid.

11. A method of producing an organic acid comprising culturing a microorganism as recited in claim 1 in the presence of an amount of an organic acid selected from the group consisting of 3HP, acrylic acid, and propionic acid.

12. The microorganism of claim 9 wherein the at least one recombinant nucleic acid encoding the 3HP pathway enzyme includes a malonyl-CoA reductase gene.

13. The microorganism of claim 9 wherein the at least one recombinant nucleic acid encoding the 3HP pathway enzyme includes an acetyl-CoA carboxylase gene.

14. The microbial culture of claim 10 wherein the amount of the organic acid is selected from the group consisting of at least about 10 mM acrylic acid, at least about 100 mM 3HP, and at least about 200 mM propionic acid.

15. The method of claim 11 wherein the amount of the organic acid is selected from the group consisting of at least about 10 mM acrylic acid, at least about 100 mM 3HP, and at least about 200 mM propionic acid.

16. A method of using acsA or homolog thereof as a counter-selectable marker comprising: replacing an acsA or homolog thereof in a bacterium with a gene of interest; and selecting for the bacterium comprising the gene of interest with an amount of an organic acid effective to inhibit growth of bacteria harboring a functional acsA gene or homolog thereof.

17. The method of claim 16 wherein the acsA or homolog thereof is an acsA gene with at least one silent nucleic acid mutation that reduces background mutation frequency.

18. The method of claim 16 wherein the at least one silent nucleic acid mutation is selected from the group consisting of T144C and G150C in acsA from Synechococcus sp. PCC 7002.

19. The method of claim 16 wherein the selecting results in the bacterium being homozygous for the gene of interest.

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