MICROORGANISMS HAVING ENHANCED RESISTANCE TO ACETATE AND RELATED COMPOSITIONS AND METHODS OF USE

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ABSTRACT

The present invention provides isolated or genetically modified strains of microorganisms that display enhanced resistance to acetate as a result of increased expression of a sodium proton antiporter. The present invention also provides methods for producing such microbial strains, as well as related promoter sequences and expression vectors. Further, the present invention provides methods of producing alcohol from biomass materials by using microorganisms with enhanced resistance to acetate.

Up F

Mutant Deletion location

MF2

MR2

Down F

Mutant deletion Loci

Down R

Up R

0116

0117

0119

0120

 terminator

 terminator
Figure 2

log2 Difference between Strains = (Mutant)-(ZM4)
Figure 4A. Nucleotide sequence of ZM4 nhaA gene.

Figure 4B. Protein sequence of ZM4 NhaA anti-porter.
0 mM NaAc (RM, pH5; AnO₂)

FIGURE 5A
195 mM NaAc (RM, pH5; AnO₂)
0 mM NaAc (RM, pH 5; O₂)

OD₆₀₀nm

0  0.1  0.2  0.3  0.4  0.5  0.6  0.7  0.8  0.9  1.0  1.1  1.2

0  5  10  15  20  25  30  35  40  45

Time (h)

FIGURE 5D
FIGURE 5E
195 mM NaAc (RM, pH 5; O₂)

FIGURE 5F

Time (h)

OD₆₀₀nm
MICROORGANISMS HAVING ENHANCED RESISTANCE TO ACETATE AND RELATED COMPOSITIONS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority from U.S. Provisional Application No. 61/173,649, filed on Apr. 29, 2009.

[0002] This invention was made with government support under Contract Number DE-AC05-00OR22725 between the United States Department of Energy and UT-Battelle, L.L.C. The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] This invention generally relates to the field of microorganism and genetic modification thereof. In particular, the invention relates to microorganisms that display enhanced resistance to acetate as a result of increased expression of an antiporter gene, and are therefore advantageous for use in fermentation of biomass materials to produce biofuels such as ethanol. Related compositions, including promoter sequences, expression vectors, genetically engineered microbial strains, as well as methods of making and using the strains, are also provided by the invention.

BACKGROUND OF THE INVENTION

[0004] Biomass-based bioenergy is crucial to meet the goal of making cellulosic biofuels cost-competitive with gasoline. Lignocellulosic materials represent an abundant feedstock for cellulosic-biofuel production. A core challenge in converting cellulosic material to biofuels such as ethanol and butanol is the recalcitrance of biomass to breakdown. Because of the complex structure of lignocellulosic biomass, pretreatment is necessary to make it accessible for enzymatic attack. Severe biomass pretreatments are required to release the sugars, which along with by-products of fermentation can create inhibitors in the production of ethanol or butanol, for example. During the pretreatment processes, a range of inhibitory chemicals are formed that include sugar degradation products such as furfural and hydroxymethyl furfural (HMF); weak acids such as acetic, formic, and levulinic acids; lignin degradation products such as the substituted phenolics vanillin and lignin monomers. In addition, the metabolic byproducts such as ethanol, lactate, and acetate also impact the fermentation by slowing and potentially stopping the fermentation prematurely. The increased lag phase and slower growth increases the ethanol cost due to both ethanol production rate and total ethanol yield decreases (Takahashi et al. 1999; Kadar et al. 2007).

[0005] Efficient conversion of lignocellulosic hydrolysates to biofuel requires high-yield production and resistance to industrially relevant stresses and inhibitors. To overcome the issue of inhibition caused by pretreatment processes, there are two approaches, one is to remove the inhibitor after pretreatment from the biomass physically or chemically, which requires extra equipment and time leading to increased costs. A second approach utilizes inhibitor tolerant microorganisms for efficient fermentation of lignocellulosic material to ethanol and their utility is considered an industrial requirement (Almeida et al. 2007).

[0006] *Z. mobilis* are gram-negative facultative anaerobic bacteria with a number of desirable industrial characteristics, such as high-specific productivity and ethanol yield, unique anaerobic use of the Entner-Doudoroff pathway that results in low cell mass formation, high ethanol tolerance (12%), pH 3.5-7.5 range for ethanol production and has a generally regarded as safe (GRAS) status (Swings and De Ley 1977; Rogers et al. 1984; Gunasekaran and Raj 1999; Dien et al. 2003; Panesar et al. 2006; Rogers et al. 2007). One drawback to using wild-type *Z. mobilis* is its narrow substrate utilization range. However, recombinant *Z. mobilis* strains have been developed to ferment pentose sugars such as xylose and arabinose (Zhang et al. 1995; Deanda et al. 1996; Mohagheghi et al. 2002). A newly formed partnership between the DuPont and Boon companies utilizes recombinant *Z. mobilis* strains for bioethanol fermentation from the lignocellulosic residues such as corn stover (Reisch 2006). On the other hand, low tolerance to acetic acid and decreased ethanol tolerance have been reported in recombinant strains (Ranatunga et al. 1997; Lawford and Rousseau 1998; Lawford et al. 2001; Dien et al. 2003).

[0007] Acetic acid is an inhibitor produced by the de-acetylation of hemicelluloses during biomass pretreatment. At pH 5.0, 36% of acetic acid is in the uncharged and undissociated form (HAc) and is able to permeate the *Z. mobilis* plasma membrane (Lawford and Rousseau 1993). The inhibition mechanism has been ascribed to the ability of the undissociated (protonated) form to cross the cell membrane leading to uncoupling and anion accumulation causing cytoplasmic acidification. Its importance comes from the significant concentrations of acetate that are produced relative to fermentable sugars (McMillan 1994) and the ratio of acetate to fermentable sugars is particularly high in material from hardwoods (Lawford and Rousseau 1993). Acetate may reach inhibitory levels when pretreated biomass hydrolysates are concentrated to generate high final ethanol concentrations or where process water is recycled. Acetate removal processes have been described but they are energy or chemical-intensive and their impact on processing costs have yet to be determined (McMillan 1994).

[0008] An acetate tolerant *Z. mobilis* mutant (AcR) has been generated by a random mutagenesis and selection strategy (Joachimsthal and Rogers 1998). The MR mutant was capable of efficient ethanol production in the presence of 20 g/L sodium acetate while the parent ZM4 was inhibited significantly above 12 g/L sodium acetate under the same conditions. A number of studies have characterized the performance of recombinant *Z. mobilis* strains able to utilize both C-5 and C-6 sugars, including under acetate stress conditions (Lawford et al. 1999; Joachimsthal and Rogers 2000; Lawford and Rousseau 2001). Acetic acid was shown to be strongly inhibitory to wild-type derived strain ZM4(pZBS) on xylose medium and nuclear magnetic resonance studies indicated intracellular deenergization and acidification appeared to be the major inhibition mechanisms (Kim et al. 2000). A recombinant strain able to utilize both xylose (α-C-5 sugar) and glucose (a C-6 sugar) with increased acetate resistance was generated by transforming plasmid pZBS into the AcR background (Jean et al. 2002). Mohagheghi et al. (2004) reported a recombinant *Zymomonas mobilis* 8b tolerant up to 16 g/L acetic acid and achieved 82%-87% (w/w) ethanol yields from pure glucose/xylose solutions.

[0009] Acetic acid bacteria are used for the industrial production of vinegar and are intrinsically resistant to acetic acid. Although the resistance mechanism is not completely understood, progress toward this goal has been made in recent
years. Spontaneous acetic acid bacteria mutants for Aceto-
bacter aceti (Okumura et al. 1985) and several Acetobacter
pasteurianus strains (Takemura et al. 1991; Chinnawirot-
pisan et al. 2003) showed growth defects in the presence of
acetic acid, which was associated with loss of alcohol dehy-
drogenase activity. Fukaya et al. (1990) identified the aarA,
aarB, and aarC gene cluster as being important for conferring
acetic acid resistance using a genetic approach (Fukaya et al.
1990). aarA encodes citrate synthase and aarC encodes a
protein that is involved in acetate assimilation (Fukaya et al.
1993) and the three aar genes have been suggested to support
increased flux through a complete but unusual citric acid
cycle to lower cytoplasmic acetate levels (Mullins et al.
2008). The presence of a proton motive force-dependent
efflux system for acetic acid has been demonstrated as being
important in A. aceti acetic acid resistance, although the
genetic determinant(s) remain to be identified (Matsushita et
al. 2005). In E. coli, over-expression of the ATP-dependent
helicase RecG has been reported to improve resistance to
weak organic acids including acetate (Steiner and Sauer
2003). Baunler et al. (2006) describe the enhancement of
acid tolerance in Z. mobilis by the expression of a proton-
buffering peptide in acidified TSB (pH 3.0) or acetic acid
(pH 3.5), glycine-HCl buffer (pH 3.0) and sodium
acetate-acetic acid buffer (pH 3.5) (Baunler et al. 2006).
Baunler et al. (2006) also note that the presence of the anti-
biotic also significantly increased acid tolerance by an
unknown mechanism.

SUMMARY OF THE INVENTION

[0010] It has been identified in accordance with the present
invention that increased expression of a sodium-proton anti-
porter gene in a microorganism confers enhanced acetate
resistance to the microorganism. In accordance with the
present invention, microorganisms can be genetically modi-
fied to increase the expression of its sodium-proton antiporter
to achieve enhanced resistance to an acetate salt (e.g., sodium,
potassium or ammonium acetate) or acetic acid. Such geneti-
cally modified microorganisms are particularly useful for
production of biofuels based on fermentation of biomass
materials.

[0011] In one aspect, the invention is directed to isolated
microorganisms that display enhanced resistance to acetate
as a result of increased expression of a sodium-proton antiporter
in the microorganisms.

[0012] In a preferred embodiment, the sodium-proton anti-
porter being expressed at an elevated level is a plasma mem-
brane sodium-proton antiporter. In other embodiments, the
sodium-proton antiporter being expressed at an elevated level
is an endosomal or vacuolar sodium-proton antiporter. The
sodium-proton antiporter being expressed at an elevated level
is preferably encoded by an nhaA gene or an nhaA homolog.

[0013] Microorganisms contemplated by the present
invention include both bacteria (including Gram-negative and
Gram positive bacterial) and fungi. Examples of bacteria of
particular interest include Acetobacterium, Bacillus, Strepto-
coccus, Clostridium, Zymomonas sp. (e.g., Z. mobilis), and
Gluconobacter sp. Examples of fungi include Saccharomy-
ces sp., Kluyveromyces sp., Pichia sp., Candida sp., and
Schizosaccharomyces sp.

[0014] In a specific embodiment, the microbial strain is a Z.
mobilis strain that displays enhanced resistance to sodium
acetate as a result of increased expression of a sodium-proton
antiporter. In another specific embodiment, the microbial
strain is a yeast strain such as a Saccharomyces or Pichia
strain, which displays enhanced resistance to one or more
acetate salts as a result of increased expression of a sodium-
proton antiporter.

[0015] In one embodiment, a microbial strain having
enhanced resistance to acetate is created by genetically modi-
fying the 5′ upstream region of the endogenous nhaA gene
of the strain.

[0016] In a specific embodiment, the microbial strain is a Z.
mobilis strain, wherein the 5′ upstream region of the nhaA
gene is modified such that a nucleotide sequence of 1000 bp
or less containing SEQ ID NO: 4 is deleted, while the nucleo-	ide sequence of SEQ ID NO: 4 immediately 5′ to the nhaA
coding sequence remains intact. Such Z. mobilis strain dis-
plays constitutive and elevated expression of nhaA, and
enhanced resistance to sodium acetate.

[0017] Isolated nucleic acids containing a nucleotide
sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or derivatives
thereof, form separate embodiments of the present invention.

[0018] In another embodiment, a microbial strain having
enhanced resistance to acetate is created by introducing an
exogenous expression vector into the strain which directs
expression (i.e., additional expression) of a sodium-proton
antiporter. Preferably, the sodium proton antiporter expressed
from the exogenous vector is identical with an endogenous
sodium proton antiporter, particularly an endogenous plasma
membrane sodium proton antiporter.

[0019] The genetically modified microorganisms that display
enhanced resistance to acetate as a result of increased expression
of a sodium-proton antiporter can be additionally modified as appropriate, for example, by transformation with
additional recombinant genes or sequences suitable for fer-
mentation and production of ethanol.

[0020] In a further aspect, the present invention provides
a method of producing biofuels from cellulosic biomass based
on use of the microbial strains that are able to grow at elevated
concentrations of acetate or acetic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 depicts a portion of the genomic sequence of
the wild type ZM4 strain, the location of the deleted portion in
AcR, and the primers used in the experiments described in
Example 1 and Example 2. 0116, 0117, 0119, and 0120
represent the genes, ZMO0116, ZMO0117, ZMO0119,
and ZMO0120, respectively. The two "terminators" are termina-
tor sites predicted by TIGR annotation. The grey line above
the ORFs and the open ORF box without an arrow referred to
as "Mutant Deletion location" is the genomic sequence
present in wild type ZM4 but deleted in AcR mutant. UP_F,
UP_R, Down_F, and Down_R are primers used for construc-
tion of the deletion mutant mimicking the AcR mutant with
the 1.5-kb deletion using the pJK100 deletion system. MF2
and MR2 are primers used to construct the insertional mutant
of ZMO0117 using the pKnock system. The primer
sequences are: MF2: ggtcagctacgagct (SEQ ID NO: 6); 
MR2: ggtcagctacgagct (SEQ ID NO: 7); UP_F: 
CGACGCTttggtagtaagactg (SEQ ID NO: 8); UP_R: 
GCCGCGGtagtaagactgagct (SEQ ID NO: 9); Down_F: 
GCATACGgtttagtaagactg (SEQ ID NO: 10); and Down_R: 
GCATACGgtttagtaagactg (SEQ ID NO: 11). Capitalized nucleotides represent a restriction enzyme site.

[0022] FIG. 2 depicts the Volcano plot results from a JMP
Genomics microarray analysis showing a summary of all
significantly differentially expressed genes between AcR and the ZM4 wild-type strain for all conditions (sodium acetate and sodium chloride in exponentially and stationary phase cells). The X-axis shows the difference values between AcR and ZM4 expression profiles based on a log2 scale. The Y-axis shows statistical significance values for expression values, based on a $-\log_{10} p$-value. The red dashed line shows the statistical significance cut-off used in this study.

[0023] FIG. 3 graphically depicts the vector map of plasmid pBR3-DEST2 constructed to analyze gene over-expressing and complementation. Tc(R): Tetracycline resistance gene tet; Cm: chloramphenicol resistance gene cat. attB1 and attR2 are recombination sites allowing recombinational cloning of the gene of interest from an entry clone; cedB is cedB gene allowing negative selection of expression clones.

[0024] FIGS. 4A-4B set forth the nucleotide sequence (SEQ ID NO: 1) and protein sequence (SEQ ID NO: 2) of ZMO0119.

[0025] FIGS. 5A-5F. The strains were grown in RM (pH 5.0) overnight, and a 0.1% culture was then transferred into 250-mL RM media in the Bioscreen plate. The growth of all strains was monitored by Bioscreen (Growth Curve USA, Piscataway, N.J.) under anaerobic (A, B, C) and aerobic (D, E, F) conditions in RM (pH 5.0) containing 0 g/L NaAc (A, D), 12 g/L NaAc (B, E), and 16 g/L NaAc (C, F) respectively. The strains included in this study are: ZM4: Zymomonas mobilis ZM4 wild-type; AcR: ZM4 acetate tolerant mutant; ZM4 (p42-0119): ZM4 containing a gateway plasmid p42-0119 over-expressing ZM4 gene ZMO0119; ZM4M0117: ZM4 insertional mutant of ZMO0117; ZM4DM0117: ZM4 deletion mutant mimicking AcR strain with a 1.5-kb deletion affecting ZMO0117 and the promoter region of ZMO0119. This experiment was repeated at least three times with similar results. Triplicates were used for each condition.

[0026] FIG. 6 shows the growth curve of wild-type Z. mobilis ZM4 and acetate tolerant mutant AcR in RM with 8.5% g/L NaCl. The data are from well-controlled anaerobic fermentation. The average of two replicate fermentors for each strain was plotted against the time point inoculation with the bar showing the standard deviation.

[0027] FIGS. 7A-7C show the effect of nhaA on growth of Z. mobilis in different forms of acetate. The growth differences of different strains were monitored by Bioscreen (Growth Curve USA) under anaerobic conditions in RM, pH 5.0 (A); RM with 195 mM NaAc, pH 5.0 (B); 195 mM NaCl, NaAc, NH4OAc, or KAc at pH 5.0 (C). This experiment was repeated at least three times with similar results. Triplicates were used for each condition.

[0028] FIGS. 8A-8D demonstrate that sodium proton antiporter proteins in S. cerevisiae are responsible for sodium acetate tolerance.

**DETAILED DESCRIPTION OF THE INVENTION**

[0029] It has been identified in accordance with the present invention that increased expression of a sodium-proton antiporter gene in a microorganism confers enhanced acetate resistance to the microorganism. The present invention provides strains of microorganisms displaying enhanced resistance to acetate, which are particularly advantageous for use in fermentation of biomass materials to produce biofuels such as ethanol and butanol. The present invention also provides methods for producing such microbial strains, as well as related novel promoter sequences and expression vectors. Further, the present invention provides methods of producing biofuels from fermentation of biomass materials by utilizing the microorganisms of the present invention.

[0030] In one aspect, the invention is directed to isolated strains of microorganisms that display enhanced resistance to acetate as a result of increased expression of a sodium-proton antiporter in the microorganism.

[0031] In one embodiment, the sodium-proton antiporter being expressed at an elevated level is a plasma membrane sodium-proton antiporter. In other embodiments, the sodium-proton antiporter being expressed at an elevated level is a sodium-proton antiporter other than a plasma membrane sodium-proton antiporter, for example, an endosomal or vacuolar sodium-proton antiporter.

[0032] The sodium-proton antiporter being expressed at an elevated level is preferably encoded by an nhaA gene or an nhaA homolog.

[0033] The name “nhaA”, as used herein and consistent with the understanding in the art, refers to a gene that encodes a plasma membrane sodium proton anti-porter in a microorganism. nhaA has been identified from a wide variety of microorganisms and the function of the encoded protein appears to be conserved based on significant sequence homologies shared across species. The nhaA gene and the encoded protein from the ZM4 strain of Zymomonas mobilis are described in FIGS. 4A-4B, and also set forth in SEQ ID NO: 1 and 2, respectively. The E. coli nhaA gene (SEQ ID NO: 12) and the encoded Na+/H+ anti-porter protein (SEQ ID NO: 13) have been extensively characterized, as summarized in the EcoliDB™ database for the Escherichia coli K-12 MG1655™, available online. Na+/H+ anti-porter genes have also been identified from various yeast species. For example, the plasma membrane Na+/H+ anti-porter has been identified from Schizosaccharomyces pombe and named as “SOD2” (Jia et al. 1992). NHA1 has been cloned from S. cerevisiae (Prior et al. 1996). The nucleotide and amino acid sequences of S. cerevisiae NHA1 are set forth in SEQ ID NO: 14 and SEQ ID NO: 15, respectively.

[0034] For purpose of the present invention, the terms “nhaA” and “nhaA homolog” together include both microbial genes that have been named as nhaA, as well as genes that have been named differently (e.g., SOD2 or NHA1) and also encode a plasma membrane sodium proton anti-porter of a microorganism that can be readily determined to be the nhaA counterpart of that microorganism based on sequence comparison, plasma membrane localization and/or functional characteristics. For example, a gene that encodes a protein that shares at least 35% identity or similarity, or preferably 40%, 45%, 50%, 60%, 75%, or 85% identity or similarity, or more preferably 90% or 95% identity or similarity, with the protein sequence of SEQ ID NO: 2 (Zymomonas mobilis), SEQ ID NO: 13 (E. coli), or SEQ ID NO: 15 (S. cerevisiae), can be considered an nhaA gene or homolog. Similarity between two protein sequences can be determined, for example, using the well known Lipman-Pearson Protein Alignment program with the following choice of parameters: Ktiple=2, Gap Penalty=4, and Gap Length Penalty=12. Alternatively, a gene that shares at least 45% identity, or preferably 50%, 60%, 75%, or 85% identity, or more preferably 90% or 95% identity with the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 12 (E. coli), or SEQ ID NO: 14 (S. cerevisiae), or a gene that hybridizes to a nucleic acid represented by any one of SEQ ID NO: 1, SEQ ID NO: 12, or SEQ ID NO: 14, or a full complement thereof, under stringent hybridization conditions, can be considered an nhaA gene or
homolog. Appropriate hybridization conditions for such determination include hybridization at 42°C to 65°C, followed by washing in 0.1x to 2x SSC, 0.1% SDS at a temperature ranging from room temperature to 65°C. More preferably but not absolutely necessary, the determination of whether a particular gene is nhaA or not is made in conjunction with confirmation that the encoded protein is localized in the plasma membrane and/or with a functional determination that the gene is involved in sodium proton exchange.

[0035] The term “nhaA homolog” also includes genes that encode a microbial sodium proton anti-porter that shares significant sequence homology to nhaA and is not necessarily a plasma membrane sodium proton antiporter; for example, an endosomal sodium proton anti-porter, or a vacuolar sodium proton anti-porter. By “significant sequence homology”, it is meant that the homolog gene encodes a protein that shares at least 35% identity or similarity, or preferably 45%, 50%, 60%, 75%, or 85% identity or similarity, or more preferably 90% or 95% identity or similarity with the protein sequence of SEQ ID NO: 2, SEQ ID NO: 13 or SEQ ID NO: 15; or alternatively, the homolog gene shares at least 45% identity, or preferably 50%, 60%, 75%, or 85% identity, or more preferably 90% or 95% identity with the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 12 or SEQ ID NO: 14, or hybridizes to a nucleic acid represented by SEQ ID NO: 1, SEQ ID NO: 12 or SEQ ID NO: 14 or the full complement thereof under stringent hybridization conditions defined above.

[0036] In accordance with the present invention, either a full length antiporter or a functional or enzymatically active fragment thereof, can be expressed at an elevated level to achieve enhanced resistance to acetate. The term “functional fragment” or “enzymatically active fragment” means a polypeptide fragment of a sodium-proton antiporter which substantially retains the activity of the full-length protein. By “substantially” it is meant at least about 50%, 60%, 70%, 80%, 90% or more of the activity of the full-length protein is retained. Based on the domain characterizations of sodium-proton antiporters and substantial consensus conserved among species, those skilled in the art can readily identify and make functional fragments of a sodium-proton antiporter using various genetic engineering techniques known in the art. The activity of any partial protein of interest can be determined using, e.g., functional complementation analysis, well known in the art.

[0037] The microbial strains of the present invention display enhanced resistance to acetate as a result of increased expression of a sodium-proton antiporter. By “increased expression”, it is meant that the level of either mRNA or protein of both of the sodium-proton antiporter is increased, preferably as a result of a genetic modification, as compared to the wild type or parental strain without the genetic modification. The extent of increase in expression contemplated by the present invention is at least 50%, or at least 100% (i.e., twice the level of parental strain), or more preferably at least four or five times, or even more preferably at least ten to fifteen times, the level of parental strain. It has been demonstrated by the present inventors that the mRNA level of nhaA in both stationary and exponentially growing cells of the Z. mobilis mutant AcR was more than sixteen (16) times the level in a wild type strain.

[0038] According to the present invention, increased expression of a sodium-proton antiporter in a microorganism, preferably a plasma membrane sodium-proton antiporter encoded by nhaA or homolog, confers enhanced resistance to acetate. By “resistance to acetate”, it is meant resistance to acetate salts including, for example, one or more of sodium acetate, potassium acetate, and ammonium acetate, and/or resistance to acetic acid. Resistance of a strain to acetate can be determined by assessing the growth of the strain in media containing various concentrations of acetate (e.g., sodium acetate) or acetic acid. By “enhanced resistance” it is meant that a strain containing a desirable genetic modification resulting in increased antiporter expression is able to grow in media containing a higher concentration of acetate (e.g., sodium acetate) or acetic acid than the unmodified strain. For example, the concentration of sodium acetate or acetic acid that can be tolerated by the strain is increased by 15%, 20%, 30%, or 50% or higher. For instance, wild type Z. mobilis strain ZM4 and the mutant strain AcR both grow well in media containing 8 g/L or even 12 g/L sodium acetate or acetic acid. However, the difference between the two strains is substantial when grown in media containing 16 g/L sodium acetate or acetic acid under anaerobic conditions. Alternatively, “enhanced resistance” can mean that the strain containing a genetic modification grows better than the unmodified strain in media containing a given concentration of sodium acetate or acetic acid, as measured by a shorter doubling time (e.g., shortened by 10%, 20%, 30% or 50% or greater) or a higher cell density reached at the end of the exponential growth phase (e.g., 25%, 50%, 75%, 100%, 150%, 200%, 500%, or even 1000% or higher cell density).

[0039] Microorganisms encompassed within the scope of the present invention include both bacteria and fungi.

[0040] In accordance with the present invention, bacterial strains having enhanced resistance to sodium acetate as a result of increased expression of nhaA include both Gram-positive and Gram-negative bacteria. Examples of Gram-positive bacteria include those from the genus of phylum Firmicutes, particularly strains of Acetobacterium, Bacillus, Streptococcus, and Clostridium. Examples of Gram-negative bacteria of particular interest include those generally considered medically safe, such as Zymomonas sp. (e.g., Z. mobilis), Glucoacetobacter sp. (e.g., Glucoacetobacter oxydans, previously known as A. suboxydans), Cyanobacteria, Gram-negative sulfur and Gram-negative non-sulfur bacteria. The acetate tolerant Z. mobilis mutant AcR described by Joachimsthal (1998) and derivatives made from this mutant (e.g., by further transformation with additional vectors in the same genetic background as the AcR mutant) are excluded from the scope of this invention. The term “same genetic background as the AcR mutant”, as used in the context of the present invention, refers to strains bearing the same genetic modification as the AcR mutant which is responsible for the phenotype of enhanced resistance to acetate.

[0041] Fungal strains having enhanced resistance to acetate as a result of increased expression of nhaA include filamentous and unicellular fungal species, particularly the species from the class of Ascomycota, for example, Saccharomyces sp., Kluyveromyces sp., Pichia sp., Candida sp., and Schizosaccharomyces sp. Preferred fungal strains contemplated by the present invention are S. cerevisiae, S. pombe, and Pichia pastoris.

[0042] Strains of microorganisms that display enhanced resistance to acetate as a result of increased expression of a sodium-proton antiporter can be made using any of the known genetic engineering techniques.
In one embodiment, a microbial strain having enhanced resistance to acetate can be created by genetically modifying the 5' upstream regulatory region of the endogenous nhaA gene of the strain.

It has been specifically demonstrated by the present inventors that a deletion of a 5' upstream sequence relative to the nhaA coding sequence in Z. mobilis results in an increased nhaA gene expression and enhanced resistance to acetate. More specifically, of the 158 nucleotides (SEQ ID NO: 3) of the nhaA promoter region, a deletion of the first 98 nucleotides (SEQIDNO:5), namely, the presence of the remaining 60 nucleotides (SEQ ID NO: 5), is sufficient to result in increased nhaA expression and enhanced acetate resistance.

In this context, the invention provides separate embodiments directed to isolated nucleic acids based on the 5' upstream regulatory elements (i.e., SEQ ID NO: 4 and 5) of the nhaA gene from Z. mobilis. Nucleic acid molecules derived from these specific elements, e.g., by substitution, addition or deletion of one or more nucleotides of SEQ ID NO: 4 or 5, are also contemplated by the present invention, so long as the modifications do not abolish the regulatory activity of SEQ ID NO: 4 or SEQ ID NO: 5. Those skilled in the art can further determine the minimal or critical element(s) within SEQ ID NO: 5 sufficient to direct elevated nhaA expression. Additionally, those skilled in the art can determine the regulatory element(s) within SEQ ID NO: 4, apparent for negatively regulating the nhaA expression. A variety of methods and techniques are available for these determinations. For example, primer extension experiments can be conducted to determine the precise transcription start site. Promoter serial deletions can be generated and tested to identify the minimum sequence required for elevated nhaA expression. DNasel footprint analysis can also be performed to identify potential repressor and activator sites within the promoter region. These regulatory elements identified in accordance with the present invention can be used independently, e.g., placed in operable linkage to a heterologous gene (a non-nhaA gene) for directing expression of such heterologous gene.

By modifying the 5' upstream region of the nhaA gene through, e.g., deletion of the first 98 nucleotides in the promoter sequence, the present invention provides a genetically modified Z. mobilis strain, wherein the modified promoter (SEQIDNO:4) by SEQIDNO:5 (60 bp). Although the genetic modification of the 5' upstream region can include sequences of ORF 0117 (i.e., the ORF 5' of the nhaA promoter), the modifications contemplated by the present invention do not include the precise modification (i.e., 1.5 kb deletion) found in the AcrR mutant. The genetic modification can include, however, a deletion of a 5' upstream sequence of less than 1.0 kb, or less than 500 bp, or about 100 bp, which deletion includes at least the deletion of the 98 bp set forth in SEQ ID NO: 4, while preferably leaving the last 60 bp (SEQ ID NO: 5) of the promoter intact.

In another embodiment, a microbial strain having enhanced resistance to acetate can be created by introducing an exogenous expression vector into the strain which directs expression (i.e., additional expression) of a sodium-proton antiporter.

In a specific embodiment, the expression vector introduced into the strain expresses a plasma membrane sodium-proton antiporter. In a preferred embodiment, the sodium-proton antiporter is encoded by an nhaA gene. In an especially preferred embodiment, the sodium-proton antiporter is encoded by an endogenous nhaA gene (i.e., an nhaA gene native to the recipient strain), even though nhaA homologs from other related species can also be introduced.

Generally, the nhaA gene is placed in an operably linkage to a promoter and a 3' termination sequence that are functional in a recipient microbial host. The promoter can be a constitutive promoter or an inducible promoter. The promoter can be a native promoter (thereby the expression vector simply introducing additional copy or copies of the nhaA expression units) or a modified promoter derived from a native promoter (such as SEQ ID NO: 5), or a heterologous promoter from a different gene. Promoters suitable for use in expression of nhaA in a bacterial host include, for example, lac promoter, T7, T3, and SP6 phage RNA polymerase promoters. Specific examples of promoters suitable for use in expression in Zymomonas species include Z. mobilis pdc promoter and adhB promoter. Specific examples of promoters suitable for use in expression in S. cerevisiae include adh1 (constitutive high expression), fbp1 (carbon source responsive), a tetracycline-repressible system based on the CaMV promoter, and the nmt1 (no message in thiamine) promoter, which may be suitable for use in expression of nhaA in a yeast strain. The above examples of promoters are well documented in the art.

A variety of vector backbones can be used for purpose of the present invention. Choices of vectors suitable for transformation and expression in bacteria and fungi have been well documented in the art. For example, numerous plasmids have been reported for transformation and expression in Zymomonas, including, e.g., pZB serial plasmids developed based on Zymomonas cryptic plasmid, as described in U.S. Pat. Nos. 5,712,133, 5,726,053, and 8,543,760, and a cloning-compatible broad-host-range destination vector described by Pelletier et al. (2008), among many others.

The expression vector can include, in addition to the nhaA expression cassette, other sequences appropriate for maintenance and selection of the vector, e.g., a selection marker gene and a replication origin. The selection marker gene can be a gene that confers resistance to antibiotics such as ampicillin resistance (Amp'), tetracycline resistance (Tet'), neomycin resistance, hygromycin resistance, and zeocin resistance (Zeoc') genes, or a gene that provides selection based on media supplement and nutrition.

The vector can be a replicative vector (such as a replicating plasmid) or, an integrative vector which mediates the introduction of a genetic sequence into a recipient cell and subsequent integration of the sequence into the host genome.

An expression vector containing an nhaA expression cassette can be introduced into a microbial host by various approaches known in the art, including transformation (e.g., chemical reagent based transformation), electroporation and conjugation.

The genetically modified strains of microorganisms that display enhanced resistance to acetate as a result of increased expression of a sodium-proton antiporter can be additionally modified as appropriate. For example, Z. mobilis strains over-expressing nhaA can be additionally modified in order to expand the range of substrates that can be utilized by the strains for efficient ethanol production. For instance, Z. mobilis strains over-expressing nhaA can also be introduced with additional genes so that the strains can ferment xylose, arabinose or other pentose sugars as the sole carbon source to
produce ethanol. See, e.g., U.S. Pat. No. 5,514,583. Additionally, yeast strains over-expressing NHA1 can be further modified to express xylose reductase, xylitol dehydrogenase and xylulokinase, and to have reduced expression of PHO13 or a PHO13 ortholog, in order to ferment xylose. See, e.g., U.S. Pat. No. 7,285,403.

[0055] The isolated or genetically modified microbial strains of the present invention are particularly useful for production of biofuels based on fermentation of biomass materials. Therefore, in a further aspect, the present invention provides a method of producing biofuels from cellulosic biomass based on use of the microbial strains of the present invention that are able to grow at elevated concentrations of acetate.

[0056] Biofuels contemplated by the present invention include particular the types of biologically produced fuels, such as bioalcohols, based on the action of microorganisms and enzymes through fermentation of biomass materials. Examples of bioalcohols include ethanol, butanol, and propanol.

[0057] In a typical cellulosic biomass to alcohol process, raw cellulosic biomass material is pretreated in order to convert, or partially convert, cellulose and hemicellulosic components into enzymatically hydrolyzable components (e.g., poly- and oligo-saccharides). The pretreatment process also serves to separate the cellulosic and hemicellulosic components from solid lignin components also present in the raw cellulosic material. The pretreatment process typically involves reacting the raw cellulosic biomass material, often as a finely divided mixture or slurry in water, with an acid, such as sulfuric acid. Other common pretreatment processes include, for example, hot water treatment, wet oxidation, steam explosion, elevated temperature (e.g., boiling), alkali treatment and/or ammonia fiber explosion. The pretreated biomass is then treated by a saccharification step in which poly- and oligo-saccharides are enzymatically hydrolyzed into simple sugars. The free sugars and/or oligosaccharides produced in the saccharification step are then subjected to fermentation conditions for the production of ethanol or butanol, for example. Fermentation can be accomplished by combining one or more fermenting microorganisms with the produced sugars under conditions suitable for fermentation.

[0058] One can also add enzyme to the fermenter to aid in the degradation of substrate to enhance alcohol production. For example, cellulase can be added to degrade cellulose to glucose simultaneously with the fermentation of glucose to ethanol by microorganisms in the same fermenter. Similarly, a hemicellulase can be added to degrade hemicellulose.

[0059] Because the pretreatment processes and by-products of fermentation can create a range of inhibitors including acetate, it is especially advantageous to utilize the genetically modified microbial strains described herein which display enhanced resistance to acetate and are able to continue fermentation despite acetate present in the fermentation broth, either in the fermentation substrate carried over from pretreatment of biomass material, or built up as a byproduct of fermentation.

[0060] For purposes of fermentation, one strain or a mixture of several strains, some or all of which display enhanced resistance to acetate, can be used.

[0061] Specific fermentation conditions can be determined by those skilled in the art, and may depend on the particular feedstock or substrates, the microorganisms chosen and the type of biofuel desired. For example, when *Zymomonas mobilis* is employed, the optimum pH conditions range from about 3.5 to about 7.5; substrate concentrations of up to about 25% (based on glucose), and even higher under certain conditions, may be used; and no oxygen is needed at any stage for microorganism survival. Agitation is not necessary but may enhance availability of substrate and diffusion of ethanol.

[0062] After fermentation, alcohol is separated from the fermentation broth by any of the many conventional techniques known to separate alcohol from aqueous solutions, including evaporation, distillation, solvent extraction and membrane separation. Particles of substrate or microorganisms may be removed before separation to enhance separation efficiency.

[0063] The present invention is further illustrated and by no means limited by the following examples.

**Example 1**

This example describes the materials and methods used in the experiments described in the subsequent examples.

**Strains and Culture Conditions**

[0065] Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured on Luria-Bertani (LB) broth or agar plates for cloning and strain maintenance. *Z. mobilis* ZM4 was obtained from the American Type Culture Collection (ATCC 31821). AcR is the *Z. mobilis* ZM4 acetate tolerant strain as described previously (Jouchimstahl et al. 1998). ZM4 and AcR were cultured in RM medium at 30°C.

[0066] *S. cerevisiae* wild-type, deletion mutant and GST-fusion ORF over-expression strains were obtained through Open Biosystems (Huntsville, Ala.). *S. cerevisiae* strains were cultured in rich media (YPD media) and minimum complete medium (CM). CM media with 2% glucose was used for *S. cerevisiae* wild-type and *S. cerevisiae* deletion mutants. CM media with 2% glucose minus uracil was used for *S. cerevisiae* GST1-over expressing strains, and 2% galactose was used to induce the GST-fusion strains.

[0067] The growth medium for *E. coli* WM3064 was supplemented with 100 μg/mL dianaminopimelic acid (DAP). Plasmid-containing strains were routinely grown with antibiotics at the following concentrations (μg/mL): kanamycin of 50 for *E. coli* and 200 for ZM4; tetracycline, 10 for *E. coli* and 20 for ZM4; and gentamycin, 10 for *E. coli*, G418 of 200 for *S. cerevisiae* YKO deletion mutants.

**PCR and DNA Manipulations**

[0068] Genomic DNA from *Z. mobilis* was isolated using a Wizard Genomic DNA purification kit, following the manufacturer’s instructions (Promega, Madison, Wis.). Purified DNA was quantified spectrophotometrically with NanoDrop 1000 System™ (Wilmington, Del.) as well as gel electrophoresis. The QIAprep Spin Miniprep™ kit and HiSpeed Plasmid Midi™ kit (Qiagen, Valencia, Calif.) were used for plasmid isolation. PCR, restriction enzyme digestion, ligation, cloning, and DNA manipulation were following essentially the standard molecular approaches. Broad-Host-Range Destination Vector pBMR3DEST42 Construction

[0069] The construction of Gateway® cloning compatible broad-host-range destination plasmid vector pBMR3DEST42 was carried out as previously described (Pelletier et al. 2008),
except that pBBRMCS-3 containing tetracycline resistance cassette was used herein instead of the previous construct pBBR5DEST42 from pBBRMCs-5 containing the gentamicin resistance cassette. Briefly, pBBR1MCS3 plasmid DNA digested with KpnI and PvuII was gel purified with Qiagen Gel purification kit (Qiagen, Valencia, Calif.) and treated with calf intestine alkaline phosphatase. The recombination region on pET-DEST42 vector DNA (Invitrogen, Carlsbad, Calif.) was PCR-amplified using the primers 42F and 42R that included KpnI and PvuII restriction sites. The gel-purified PCR product was ligated with pBBR1MCS3 KpnI/PvuII fragment with Fast-Link™ DNA Ligation Kit (Epicentre, Madison, Wis.). Ligation products were transformed into E. coli DB3.1 chemically competent cells (Invitrogen, Carlsbad, Calif.) and the transformants were selected by plating on LB agar plates containing tetracycline. Individual colonies were grown overnight in LB containing 30 µg/mL chloramphenicol and 10 µg/mL tetracycline, and plasmid DNA was prepared using QIAprep Spin Miniprep™ or HiSpeed Plasmid MidiKit following the manufacturer’s protocol (Qiagen, Valencia, Calif.). Plasmid DNA was digested with KpnI and PvuII and digestion products were analyzed on an agarose gel to confirm the presence of products of the expected sizes.

ZMO0119 Gateway Entry Vector and Expression Clone Construction

**[0070]** The construction of entry vector and expression clone of target gene nhaA (ZMO0119) was carried out as described previously (Pelletier et al. 2008). Briefly, target gene nhaA (ZMO0119) was PCR amplified using AcrC genomic DNA as template and primer nhaA_CF and nhaA_CR as primers. PCR products were then cloned into Gateway® entry clone pDONR221 using BP Clonase II enzyme mix following the manufacturer’s protocol (Invitrogen, Carlsbad, Calif.), and then transformed into chemically competent DH5α cells (Invitrogen, Carlsbad, Calif.) and plated onto LB with appropriate antibiotic selection. The inserts were confirmed by sequencing using M13 forward and reverse primers (Integrated DNA Technologies, Inc., Coralville, Iowa). The confirmed entry clone vector was then recombined with the destination vector pBBR3DEST42 using LR Clonase II™ enzyme mix (Invitrogen Carlsbad, Calif.) to create the expression vector. The resulting expression vector construct was designed as p42-0119. The plasmid construct p42-0119 was confirmed by sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, Calif.).

Mutant Plasmid Construction

**[0071]** For the insertional hcp mutant construction, the internal part of Z. mobilis hcp gene (ZMO0117) was amplified by PCR using MF2 and MR2 supplied by MWG-Biotech (Huntsville, Ala.). The hcp and nhaA gene as well as the primer positions used for mutant construction and nhaA gene-expressing vector are shown in FIG. 1. The 529-bp hcp internal part PCR product was then purified and cloned into pCR2.1-TOPO and then transformed into E. coli TOPO one competent cell (Invitrogen, Carlsbad, Calif.). The transformants with correct construct were confirmed by PCR and sequencing. The plasmid was then extracted using a Qiagen Midiprep™ kit and digested with XbaI and HindIII restriction enzymes, the 529-bp hcp internal part was then purified by Qiagen Gel purification kit. Similarly, pKnock-Km suicide vector was also digested with XbaI and HindIII restriction enzyme following de-phosphorylation, and then ligated with 529-bp purified hcp internal part using Fast-Link™ DNA Ligation Kit (Epicentre, Madison, Wis.). The ligation product was then transformed into TransforMax EC100D pir-116 Electrocompetent E. coli competent cells (Epicentre, Madison, Wis.) by electroporation. The transformants containing plasmid pKm-0117 was selected on LB agar plate with 50 µg/mL kanamycin. The plasmid was then extracted from the transformant cells and the plasmid construct named as pKm-0117 was then sequenced to confirm the presence of the target gene fragment, which was then electropropated into E. coli WM3064 strain. The transformant E. coli WM3064 (pKm-0117) was verified by PCR and sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, Calif.) for the presence of correct plasmid construct pKm-0117.

**[0072]** For the full deletion of most of the hcp gene and part of the nhaA gene promoter region to reconstruct the ZM4 wild-type strain to mimic the Acr strain 1.5-kb deletion region (See FIG. 1), the pJK100 plasmid was used for mutant plasmid construction as described previously (Deneef et al. 2006). The primers used to amplify the upstream region (UP_F/UP_R) and downstream region (DOWN_F/DOWN_R) for mutant plasmid construction were listed in Table 1, which generated an 805 and 1050-bp PCR fragment. The final plasmid construct was named as pJcHcp_nhaA.

Plasmid Transformation of Z. mobilis

**[0073]** Z. mobilis wild-type ZM4 and acetate tolerant strain AcR cultures were grown aerobically at 30°C in RM, and E. coli WM3064 containing plasmid pKm-0117, pJK_hcp_nhaA, or p42-0119 cultures were grown at 37°C in LB containing 100 µg/mL DAP and 10 µg/mL tetracycline to exponential phase. E. coli WM3064 cells containing plasmid pKm-0117, pJK_hcp_nhaA, or p42-0119 were washed with RM for three times by centrifugation at 13,000 rpm for 1 min and resuspended in RM. Different ratio of ZM4 cells with E. coli WM3064 (pKm-0117) cells, E. coli WM3064 (pJK_hcp_nhaA), E. coli WM3064 (p42-0119) cells were mixed in different ratio (1:3, 1:1, and 3:1) and plated onto RM agar plates with 100 µg/mL DAP and 10 µg/mL tetracycline for plasmid p42-0119 conjugation or 50 µg/mL kanamycin for plasmid pKm-0117 or pJK_hcp_nhaA conjugation. The cells were incubated at 30°C overnight. Conjugants were selected by plating on RM agar plates containing 20 µg/mL tetracycline for p42-0119 plasmid conjugants or 200 µg/mL kanamycin for pKm-0117 or pJK_hcp_nhaA plasmid conjugants at 30°C. The conjugants were confirmed for the presence of correct plasmid constructs by PCR and sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, Calif.).

Z. mobilis ZM4 Ultrafast Genomic Pyrosequencing

**[0074]** Ultrafast pyrosequencing using the Roche 454 Genome Sequencer FLX System (454 Life Sciences, Branford, Conn.) was carried on according to manufacturer’s instructions, and the GS FLX shotgun DNA library preparation method manual (454 Life Sciences, Branford, Conn.). Briefly, for Z. mobilis ZM4 genome resequencing, shotgun DNA library was prepared using the mechanically sheared Z. mobilis ZM4 genomic DNA fragments with specific A and B adaptors blunt end ligated. After adaptor ligation, the fragments were denatured and bluntly amplified via emulsion PCR generating millions of copies of template per bead. The DNA beads were then distributed into picolitre-sized wells on
a fibre-optic slide (PicoTiterPlate™), along with a mixture of smaller beads coated with the enzymes required for the pyrosequencing reaction, including the firefly enzyme luciferase. The four DNA nucleotides were then flushed sequentially over the plate. Light signals released upon base incorporation were captured by a CCD camera, and the sequence of bases incorporated per well was stored as a read.

Long paired-end DNA library was prepared using the same genomic DNA for shot-gun DNA library preparation following the 454 GS FLX long paired end library preparation method manual (454 Life Sciences, Branford, Conn.). Long paired-end DNA library reads were used to build the original contigs and to assemble the contigs into scaffolds. Briefly, DNA was sheared into ~3 kb fragments using hydro shear (Genomic Solutions Inc., Ann Arbor, Mich.), EcoRI restriction sites were protected via methylation, and biotinylated hairpin adaptors (containing an EcoRI site) were ligated to the fragment ends. The fragments were subjected to EcoRI digestion and circularized by ligation of the compatible ends, and subsequently randomly sheared. Biotinylated linker containing fragments were isolated by streptavidin-affinity purification. These fragments were then subjected to the standard 454 sequencing on the GS FLX system.

Pyrosequencing Data Analysis and Access

GS reference Mapper, one application from 454 Genome Sequencer FLX software package 1.1.03 (454 Life Sciences, Branford, Conn.), was used to map the reads generated from GS sequencer application onto the Z. mobilis ZM4 reference genome (GenBank accession: AE008692) and reference plasmids Z. mobilis ZM4 plasmid 1 (GenBank accession: AY057845) as described in the GS FLX Data Analysis Software Manual (454 Life Sciences, Branford, Conn.). Sequence reads, contigs, and quality scores for nucleotide sequences and contigs were provided by 454 Life Science. High quality differences in which the identity frequency of all reads greater than 85% were used to change the genome sequence prior to final annotation. De novo Assembler, another application from 454 Genome Sequencer FLX software package was used to assemble the sequence reads into contigs. The contigs generated were then compared with ZM4 genome sequence and the plasmid sequence deposited in GenBank (AY05748). Contigs belong to plasmid sequence were aligned to the reference sequence and the contig gaps in original deposit were corrected, which gave a full-length plasmid sequence.

Microarray Transcriptomic RNA Profiling:

Overall design.—Whole genome expression profiles of exponential and stationary phase cells were analyzed for the wild-type Zymomonas mobilis ZM4 and acetate tolerant mutant AcR under 12 g/L sodium acetate and same molar concentration of sodium chloride (8.55 g/L) control conditions.

Growth protocol.—Z. mobilis ZM4 was obtained from the American Type Culture Collection (ATCC31821) and cultured in RM medium at 30°C. For the inoculum preparation a single colony of ZM4 was added to a test tube containing 5 ml RM broth and cultured aerobically at 30°C.

until it reached late exponential or early stationary phase. A 1/100 dilution was added into the pre-warmed RM broth (10 ml culture into 1000 ml RM), which was then cultured aerobically at 30°C with shaking at 150 rpm for approximately 12 h. The optical density was measured with a spectrophotometer at 600 nm and the inoculum was added to each fermentor so that the initial OD600 nm was approximately same in each fermentor. Batch fermentations were conducted in approximately 2.5 L of RM medium in 7.5-L BioFlo110 bioreactors (New Brunswick Scientific, Edison, N.J.) fitted with agitation, pH, temperature and DOT probes and controls. Culture pH was monitored using a pH electrode (Metler-Toledo, Columbus, Ohio) and the pH control set point was maintained at 5.0 by automatic titration with 3 N KOH. Temperature was maintained automatically at 30°C and the vented gases exiting fermentors were passed through condenser units, chilled by a NESLAB Merlin M-150 refrigerated recirculator (Thermo Fisher Scientific, Newington, N.H.) to a vented hood via a water trap. DOT was monitored by using Inh'po 6800 series polarographic O2 sensors (Metler-Toledo). Three anaerobic fermentors were sparged overnight with filter-sterilized N2 gas and for approximately one hour post-inoculation and the three aerobic fermentors were continually sparged with filter-sterilized air at 2.5 L/min to maintain fully aerobic conditions. The agitation rate was 700 rpm in each vessel.

RNA extraction protocol.—RNA was isolated essentially described previously [26]. Briefly, samples from aerobic and anaerobic fermentors were harvested by centrifugation and the TRizol reagent (Invitrogen, Carlsbad, Calif.) was used to extract total cellular RNA. Each total RNA preparation was treated with RNase-free DNase I (Ambion, Austin, Tex.) to digest residual chromosomal DNA and subsequently purified with the Qiagen RNeasy Mini kit in accordance with the instructions from the manufacturer. Total cellular RNA was quantified at OD260 and OD280 with a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del.). The purified RNA from each sample was used as the template to generate ds-cDNA using Invitrogen ds-cDNA synthesis kit (Invitrogen, Calif.).

Label, hybridization, and scan protocol: The ds-cDNA was sent to NimbleGen for labelling, hybridization, and scanning following company’s protocols.

Bioscreen Assay

Growth was monitored turbidometrically by measuring optical density at 600 nm intermittently with Bioscreen C Automated Microbiology Growth Curve Analysis System (growth curve USA, Piscataway, N.J.). For the inoculum preparation, a single colony of ZM4 was added to a test tube containing 5 ml RM (pH5.0) broth and cultured aerobically at 30°C until it reached exponential phase. Twenty-μl culture was then transferred into 250-μl RM media in the Bioscreen C plate. The growth differences of different strains were monitored by Bioscreen C under both aerobic and anaerobic conditions in RM (pH5.0). Each experiment has been repeated at least three times. Replicates were used for each condition.
### TABLE 1

**Bacterial strains, plasmids and primers used in this application**

<table>
<thead>
<tr>
<th>Strain, plasmid, or primer</th>
<th>Genotype, phenotype, or sequence of primer (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-12</td>
<td>K-12 MG1655 Wild-type strain</td>
<td>Joachimstahl et al. (1998)</td>
</tr>
<tr>
<td>DH5α</td>
<td>F' φ80d/acidZM15 λlacZY A-argF:U169 recA1 endA1 hsdR17(rK-, mK+) phoA supE44 Δ thi-1 gyr96 relA1</td>
<td>Novagen</td>
</tr>
<tr>
<td>DB3.1</td>
<td>F' gyrA462 endA1(prrl-reca) mcrB mrr hsdS28(rK-, mK+) Invitrogen</td>
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</tr>
<tr>
<td>N3064</td>
<td></td>
<td>Denef et al. (2006)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>P-ompT hsdS4(rB-mB-) gal dcm (DE3)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Zymomonas mobilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZM4</td>
<td>ATCC11821 ZM4 acetate tolerant strain generated by random mutagenesis</td>
<td>Joachimstahl et al. (1998)</td>
</tr>
<tr>
<td>AcR</td>
<td>ZM4 containing plasmid p42-0117</td>
<td>This application</td>
</tr>
<tr>
<td>ZM4[p42-0119]</td>
<td>Insertional mutant of ZM4 gene W00117</td>
<td>This application</td>
</tr>
<tr>
<td>ZM4ΔMO0117</td>
<td>Deletion mutant of ZM4 gene W00117 and part of W00119 promoter region to mimic the AcR deletion region</td>
<td>This application</td>
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<td><strong>S. cerevisiae</strong></td>
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</tr>
<tr>
<td>BY4741</td>
<td>MATa his3A1 leu2A0 ura3A0 met15A0-c288c Open background</td>
<td>Biosystems</td>
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<td>YSC1021-5</td>
<td>Yeast: Yeast Knock Out Strain, NHA1 Open</td>
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<td>552692</td>
<td>Clone Id: 4095 Accession: YLR138W</td>
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<td>YSC1021-1</td>
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<td>Open</td>
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<td>Clone Id: 1123 Accession: YNL321W</td>
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<td>YSC1021-2</td>
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<td>YSC4515-1</td>
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<td><strong>Plasmids</strong></td>
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<td>Alexeyev et al. (1999)</td>
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<td>pXK100</td>
<td>Em', mob, broad host range suicide vector</td>
<td>Denef et al. (2006)</td>
</tr>
<tr>
<td>pKT-DEST42</td>
<td>Ap', Cm', C-terminal 6XHis and V5 epitope</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBBR1MCS-3</td>
<td>To', mob, broad host range cloning vector</td>
<td>This application</td>
</tr>
<tr>
<td>pBBR3DEST42</td>
<td>Cm' To', C-terminal 6XHis and V5 epitope</td>
<td>This application</td>
</tr>
<tr>
<td>pDNR221</td>
<td>Km', gateway entry vector Km', N-terminal GST</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>p42-0119</td>
<td>pBBR3DEST42 containing ZM4 gene ZMO0119</td>
<td>This application</td>
</tr>
<tr>
<td><strong>Primers</strong></td>
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<tr>
<td>MP2</td>
<td>gtagctacgctacgaagctgttt (SEQ ID NO: 6) 529-bp</td>
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<tr>
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<td>Down_R</td>
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</table>
Example 2

This example describes the results from the experiments conducted to determine the genetic basis for the acetate tolerance observed with the mutant Z. mobilis strain, AcR.

Using microarray comparative genome sequencing, next generation 454-pyrosequencing, and Sanger sequencing approaches, it was identified and confirmed that the genomic differences between the wild-type Z. mobilis strain, ZM4, and the acetate mutant AcR strain. The genetic changes in the mutant included a 1.5-kb deletion (FIG. 1) and single nucleotide polymorphisms (SNPs). Expression of the nhaA sodium proton anti-porter gene in AcR was found to be constitutive and significantly higher than in the wild-type strain under all the conditions tested (FIG. 2). Whole genome expression profiles were analyzed for mutant and wild-type exponential and stationary phase cells under sodium acetate and sodium chloride control conditions. A summary of these data is presented in FIG. 2 and Table 2.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Product Description</th>
<th>AcR_Exp/ ZM4_Exp</th>
<th>AcR_Stationary/ ZM4_Stationary</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZM00117</td>
<td>Hybrid cluster protein</td>
<td>-2.15</td>
<td>-2.20</td>
</tr>
<tr>
<td>ZM00119</td>
<td>Na+/H+ antiporter NhaA</td>
<td>4.60</td>
<td>4.27</td>
</tr>
<tr>
<td>ZM00120</td>
<td>Dihydroorotate dehydrogenase</td>
<td>1.71</td>
<td>0.97</td>
</tr>
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</table>

AcR_Exp/ ZM4_Exp: the ratio of gene expression between acetate tolerant mutant AcR in exponential phase and wild-type ZM4 in exponential phase.
AcR_Stationary/ ZM4_Stationary: the ratio of gene expression between acetate tolerant mutant AcR in stationary phase and wild-type ZM4 in stationary phase.
The numbers are log2 base.

Example 3

This example describes experiments conducted to test the hypothesis that the 1.5-kb deletion in the AcR genome resulted in increased nhaA expression that conferred increased acetate tolerance in the mutant.

A new Gateway® cloning compatible vector pHBR3-DFST42 was constructed. This vector contained the tetracycline resistance gene (FIG. 3) for candidate gene overexpression in ZM4 due to intrinsic broad Z. mobilis antibiotic resistance. The anti-porter nhaA gene (FIG. 4) was cloned into the vector resulting in a nhaA over-expression vector p42-019, which was then transformed into the wild-type ZM4 strain through conjugation to generate a strain over-expressing nhaA, which was named as “ZM4(p42-019)”. In addition, a deletion mutant was constructed to mimic the 1.5-kb deletion region (FIG. 1) of the AcR acetate tolerant strain using the pJK100 system (Denef et al. 2006). Since the deletion covers most of the hypothetical protein ZM00117 and the promoter region of ZM00119 (nhaA) gene (FIG. 1), an insertional mutant of ZM00117 was created using the pKNOCK system (Alexeyev 1999) to investigate the relationship between gene ZM00117 and acetate tolerance.

The wild-type ZM4 strain, the acetate mutant AcR, the 1.5-kb deletion mutant ZM4DM0117, the insertional mutant ZM4IM0117, and the nhaA over-expression strain ZM4(p42-019) were used in a BIOSCREENCTM automated microbiology growth curve analysis system to test their susceptibilities to sodium acetate stress. All strains grew similarly under anaerobic and aerobic condition in the absence of sodium acetate (FIGS. 5A and 5D). However, in the presence of 12 g/L (146 mM) sodium acetate, ZM4 wild-type and the ZM4IM0117 grew more slowly, but ZM4DM0117 and ZM4 (p42-019) were more resistant to sodium acetate stress (FIG. 5B) under anaerobic conditions. The ZM4 wild-type strain and acetate tolerant mutant AcR grew similarly with the presence of same molar amount of sodium ion (8.6 g/L sodium chloride), which indicated that nhaA was responsible for acetate tolerance of AcR (FIG. 6). The difference was more dramatic when the sodium acetate concentration was increased to 16 g/L (196 mM), with the growth of both ZM4 and ZM4IM0117 almost completely inhibited, while ZM4DM0117 and ZM4(p42-019) were able to grow well (FIG. 5C). Stressors other than sodium acetate, such as reactive oxygen species and toxic end-products, may be present under aerobic conditions (Yang et al. 2009) and over-expression of nhaA also aided the ability of ZM4 to grow under these conditions although at lower concentrations than during that of anaerobic growth. For example, ZM4 wild-type and ZM4IM0117 grew very poorly or not at all in the presence of 12 g/L sodium acetate while strains ZM4DM0117 and ZM4 (p42-019) grew well (FIG. 5E).

These results indicate that over-expression of the nhaA gene in ZM4 rendered ZM4 tolerant to sodium acetate and gene ZM00117 was not responsible for the sodium acetate tolerance.

The promoter sequence (158-bp) of the nhaA gene in the wild type ZM4 strain is:

```
(SEQ ID NO: 3)
Ttaggtcaagagttttatatatttttaaggggaaggggggttttggt
ccctttttggttatcttgaagggggtgttatatatcaaaaaagggggcga
tattanacatatacgttqtttccatattttttatcattctatcataag
ogcagctat.
```

The portion of the nhaA gene having been deleted in the AcR mutant (98-bp):

```
(SEQ ID NO: 4)
Ttaggtcaagagttttatatatttttaaggggaaggggggttttggt
cctttttggttatcttgaagggggtgttatatatcaaaaaaggg
```

The promoter sequence of nhaA remaining in AcR (60-bp):

```
(SEQ ID NO: 5)
cctttttggttatcttgaagggggtgttatatatcaaaaaagggggcga
```

Example 4

This example describes experiments conducted to investigate the role of nhaA on different forms of acetate.

ZM4 and AcR strains were grown in the presence of the same molar concentrations (195 mM) of sodium chloride (NaCl), sodium acetate (NaAc), potassium acetate (KAc), or ammonium acetate (NH4OAc). Both the sodium and acetate ions had a toxic effect on Z. mobilis growth, with decreases in
both growth rate and final cell density (Table 3; FIG. 7). The acetate ion was more toxic than the sodium ion: Z. mobilis grew more rapidly in the presence of 195 mM NaCl and the final cell density was higher compared to growth in the presence of same molar concentration of NH₄OAc or KAc (Table 3). NaAc was more inhibitory than the same molar concentration (195 mM) of KAc or NH₄OAc for ZM4 and the combination of elevated Na+ and Ac– ions appeared to exert a synergistic inhibitory effect for strain ZM4 with the growth of Z. mobilis totally inhibited (Table 3; FIG. 7). The Ac– strain was selected for sodium acetate tolerance, but also had enhanced tolerance to NaCl, but not NH₄OAc or KAc compared to the Z. mobilis wild-type ZM4 (Table 3; FIG. 7). Strain ZM4DM0117 and ZM4 harboring the nhaA-expressing plasmid p42-0119 similarly had enhanced NaCl tolerance that did not extend to NH₄OAc or KAc (Table 3; FIG. 7). The increased tolerance to NaAc for these strains may therefore be due mostly to an increased sodium ion tolerance arising from the overexpression of Na+/H+ antiporter gene nhaA.

### TABLE 3

<table>
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<tr>
<th>Growth rate (hour⁻¹)</th>
<th>RM</th>
<th>ZM4 (NaCl, 195 mM)</th>
<th>RM (NH₄OAc, 195 mM)</th>
<th>RM (KAc, 195 mM)</th>
<th>RM (NaAc, 195 mM)</th>
<th>RM (NaAc, 146 mM)</th>
<th>Final</th>
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<td></td>
<td>0.42 ± 0.01</td>
<td>0.39 ± 0.01</td>
<td>0.39 ± 0.009</td>
<td>0.40 ± 0.00</td>
<td>0.39 ± 0.004</td>
<td>0.40 ± 0.00</td>
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<tr>
<td>Density (OD₆₀₀₉)</td>
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<td>0.32 ± 0.01</td>
<td>0.28 ± 0.008</td>
<td>0.26 ± 0.008</td>
<td>0.29 ± 0.01</td>
<td>0.33 ± 0.002</td>
<td>0.28 ± 0.008</td>
<td>0.29 ± 0.01</td>
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<td>RM (NaCl, 195 mM)</td>
<td>0.73 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>0.88 ± 0.002</td>
<td>0.70 ± 0.02</td>
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<tr>
<td>KM (KAc, 195 mM)</td>
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<td>0.36 ± 0.03</td>
<td>0.40 ± 0.001</td>
<td>0.34 ± 0.003</td>
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<tr>
<td>KM (NaAc, 195 mM)</td>
<td>0.42 ± 0.002</td>
<td>0.40 ± 0.000</td>
<td>0.41 ± 0.001</td>
<td>0.40 ± 0.001</td>
<td>0.34 ± 0.003</td>
<td>0.40 ± 0.001</td>
<td>0.34 ± 0.003</td>
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</table>

*Na* indicates that the data are not available due to the lack of growth in that condition. The concentration for all the chemicals supplemented into the RM is the number after the chemical. NaCl: sodium chloride, NH₄OAc: ammonium acetate, KAc: potassium acetate, NaAc: sodium acetate. This experiment has been repeated at least three times with similar result. Duplicates biological replicates were used for each condition.

### Example 5

**[0093]** This example describes experiments conducted to examine the function of sodium/proton antiporters in yeast *S. cerevisiae*.

**[0094]** Yeast has three sodium/proton antiporters. NHA1 (YLR138W) is a Na+/H+ antiporter involved in sodium and potassium efflux through the plasma membrane; required for alkali cation tolerance at acidic pH. VNX1 (YNJ321W) is a vacuolar Na+/H+ exchanger. NHX1 (YDR456W) is an endosomal Na+/H+ exchanger, required for intracellular sequestering of Na+, and required for osmotic tolerance to acute hypertonic shock.

**[0095]** *S. cerevisiae* deletion mutants of the above three Na+/H+ exchangers and an NHA1-overexpression strain were obtained from Open Biosystems company (Table 1). The growth curves of the mutants, NHA1-overexpression strain and the wild-type strain cultured in a rich YPD media and minimum complete medium (CM) containing NaAc, NH₄OAc, or KAc were tested using the Bioscreen system. Specifically, *S. cerevisiae* strains were grown in CM with 2% glucose for wild-type BY4741 and the deletion mutants, CM with 2% glucose minus uracil for GSH over-expression strains with 2% galactose added for gene induction. A 5-μL culture was then transferred into 300-μL CM broth in the Bioscreen plate. The growth differences among different strains were monitored by Bioscreen (Growth Curve USA, N.J.). Strains included in this experiment are listed in Table 1. This experiment has been repeated at least three times with similar result.

**[0096]** As shown in FIGS. 8A-8D, each Na+/H+ antiporter mutant was more sensitive to acetate than the wild-type control strain, and tolerance to each of NaAc, NH₄OAc, and KAc could be enhanced by expression of plasmid encoded yeast Na+/H+ antiporter genes. These data indicate that Na+/H+ antiporter genes in *S. cerevisiae* are involved in acetate tolerance, and are consistent with earlier reports that these yeast systems can function as monovalent cation/H+ antiporters (Banuelos et al. 1998; Cagnac et al. 2007).

### REFERENCES


involved in catabolic acetate production, while NAD-dependent alcohol dehydrogenase in ethanol assimilation in


50. U.S. Pat. No. 5,712,133
51. U.S. Pat. No. 5,726,053
52. U.S. Pat. No. 5,843,760
53. U.S. Pat. No. 5,514,583
54. U.S. Pat. No. 7,285,403

SEQUENCE LISTING

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Ser Ala Leu Asp Ala Pro Asn Leu Ala Glu Phe Ile Ser Ile Ala
50    55     60
Pro Met Ser Leu Phe Phe Phe Val Val Ile Ala Glu Ile Lys Glu
65    70     75     80
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85    90     95
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145   150    155    160
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Pro Trp Val Thr Trp Leu Ile Leu Pro Leu Phe Gly Phe Val Ser Met
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Gly Met Ser Leu Ser Ala Met Ser Phe His Val Leu Ala Pro Val
275   280    285
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Phe Gly Ala Thr Ile Met Ala Thr Arg Leu Lys Ile Ala Thr Leu Pro
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-continued

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Alanine Threonine Serine Glycine Tryptophan Histidine Aspartic Acid Phenylalanine Leucine Glutamic Acid Threonine

Arginine Valine Glycine Tryptophan Tyrosine Aspartic Acid Phenylalanine Leucine Glutamic Acid Thrreonine Proline Valine Glutamic Acid Leucine Glutamine

Arginine Valine Leucine Methionine Alanine Valine Phenylalanine Leucine Valine Glutamic Acid Leucine Valine Leucine Arginine Glutamic Acid Serine Leucine Alanine Serine Leucine Arginine Glutamic Acid Leucine Asparagine

Proline Valine Isoleucine Alanine Isoleucine Glycine Methionine Isoleucine Valine Proline Alanine Leucine Leucine Tyrosine

Leucine Alanine Phenylalanine Tyrosine Alanine Aspartic Acid Isoleucine Threonine Arginine Glutamic Acid Tryptophan Alanine Leucine

Proline Alanine Alanine Threonine Asparagine Isoleucine Alanine Leucine Glutamic Acid Valine Leucine Leucine Leucine

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Isoleucine Alanine Aspartic Acid Leucine Glycine Isoleucine Isoleucine Alanine Leucine Phenylalanine Threonine

Asparagine Leucine Serine Met Alanine Serine Leucine Glycine Valine Alanine Valine Alanine Isoleucine

Valine Leucine Alanine Valine Lysine Cysteine Alanine Arginine Arginine Threonine Glycine Tryptophan

Isoleucine Leucine Valine Leucine Tryptophan Threonine Alanine Leucine Lysine Serine Glycine Valine

Histidine Alanine Leucine Valine Glycine Phenylalanine Tryptophan Proline Leucine Lysine

Glutamic Acid Histidine Glycine Aspartic Acid Proline Alanine Lysine Arginine Glutamic Acid Histidine Valine Leucine Histidine

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Val Pro Pro Pro Val Asp Glu Gly Ala Ile Glu Gly Pro Ser Arg 850 855 860
What is claimed is:

1. A genetically modified microorganism, wherein the genetic modification results in increased expression of a sodium-proton antiporter in said microorganism and elevated resistance to acetate as compared to microorganism without the genetic modification.

2. The microorganism of claim 1, wherein said microorganism is selected from bacteria or fungi.

3. The microorganism of claim 2, wherein said microorganism is a bacterial species selected from *Acetobacterium*, *Bacillus*, *Streptococcus*, *Clostridium*, *Lysinmonas* sp., and *Gluconobacter* sp.

4. The microorganism of claim 2, wherein said microorganism is a fungal species selected from *Saccharomyces* sp., *Kluyveromyces* sp., *Pichia* sp., *Candida* sp., and *Schizosaccharomyces* sp.

5. The microorganism of claim 1, wherein said sodium-proton antiporter is selected from the group consisting of a plasma membrane sodium-proton antiporter, an endosomal sodium-proton antiporter, and a vacuolar sodium-proton antiporter.

6. The microorganism of claim 5, wherein said sodium-proton antiporter is a plasma membrane sodium-proton antiporter native to said microorganism.

7. The microorganism of claim 1, wherein said microorganism is *Z. mobilis*.

8. The microorganism of claim 7, wherein said sodium-proton antiporter comprises the protein sequence as set forth in SEQ ID NO: 2.

9. The microorganism of claim 8, wherein the genetic modification comprises a deletion in the 5′ region upstream of the genomic coding sequence of said antiporter, wherein said deletion is less than 1000 bp and comprises SEQ ID NO: 4, and wherein the modified 5′ region comprises SEQ ID NO: 5.

10. The microorganism of claim 8, wherein said sodium-proton antiporter is expressed from an expression vector introduced into said microorganism.

11. The microorganism of claim 1, wherein said microorganism is *S. cerevisiae*.

12. The microorganism of claim 11, wherein said sodium-proton antiporter is the plasma membrane sodium-proton antiporter comprising the amino acid sequence of SEQ ID NO: 15.

13. The microorganism of claim 12, wherein said plasma membrane sodium-proton antiporter is expressed from an expression vector introduced into said *S. cerevisiae*.

14. A method of enhancing resistance of a microorganism to acetate, comprising genetically modifying said microorganism to increase expression of a sodium-proton antiporter in said microorganism.

15. The method of claim 14, wherein the genetic modification comprises modification of the 5′ region upstream of the genomic coding sequence of said sodium-proton antiporter.

16. The method of claim 14, wherein the genetic modification comprises introducing an expression vector into said microorganism, wherein said expression vector directs expression of said sodium-proton antiporter in said microorganism.

17. The method of claim 14, wherein said sodium-proton antiporter is a plasma membrane sodium-proton antiporter.

18. The method of claim 14, said microorganism is selected from bacteria or fungi.

19. An isolated nucleic acid molecule, comprising the nucleotide sequence of SEQ ID NO: 4.

20. An isolated nucleic acid molecule, comprising the nucleotide sequence of SEQ ID NO: 5.

21. A method of producing alcohol from a cellulosic biomass material, comprising adding a genetically modified microorganism according to any one of claims 1-13 to a fermentation mixture comprising a cellulosic biomass material and/or fermentation substrates derived from said cellulosic biomass material, allowing said microorganism to ferment and produce alcohol, and recovering alcohol produced.

* * * * *