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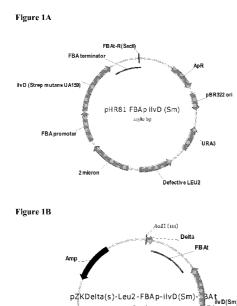
[Continued on next page]

(54) Title: IMPROVING ACTIVITY OF FE-S CLUSTER REQUIRING PROTEINS

(57) Abstract: The present invention is related to a recombinant host cell, in particular a yeast cell, comprising a dihydroxy-acid dehydratase polypeptide. The invention is also related to a recombinant host cell having increased specific activity of the dihydroxy-acid dehydratase polypeptide as a result of increased expression of the polypeptide, modulation of the Fe-S cluster biosynthesis of the cell, or a combination thereof. The present invention also includes methods of using the host cells, as well as, methods for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell.

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IMPROVING ACTIVITY OF FE-S CLUSTER REQUIRING PROTEINS

Cross-Reference to Related Applications

[0001] This application claims the benefit of U.S. Provisional Appl. No. 61/305,333, filed February 17, 2010, which is incorporated by reference in its entirety.

Sequence Listing Information

[0002] The content of the electronically submitted sequence listing in ASCII text file CL4842sequencelisting.txt filed with the application is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] This invention relates generally to the fields of microbiology and biochemistry. Specifically, the present invention is related to a recombinant host cell, in particular a yeast cell, comprising a dihydroxy-acid dehydratase polypeptide. The invention is also related to a recombinant host cell having increased specific activity of the dihydroxy-acid dehydratase polypeptide as a result of increased expression of the polypeptide, modulation of the Fe-S cluster biosynthesis activity of the cell, or a combination thereof. The present invention also includes methods of using the host cells, as well as methods for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell.

Background of the Invention

[0004] Iron-sulfur (Fe-S) clusters serve as cofactors or prosthetic groups essential for the normal function of the class of proteins that contain them. In the class of Fe-S cluster containing proteins, the Fe-S clusters have been found to play several roles. When proteins of this class are first synthesized by the cell, they lack the Fe-S clusters required for their proper function and are referred to as apoproteins. Fe-S clusters are made in a series of reactions by proteins involved in Fe-S cluster biosynthesis and are transferred to the apo-proteins to form the functional Fe-S cluster containing holoproteins.

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- [0005] One such protein that requires Fe-S clusters for proper function is dihydroxy-acid dehydratase (DHAD) (E.C. 4.2.1.9). DHAD catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate, and of 2,3-dihydroxymethylvalerate to α-ketomethylvalerate. The DHAD enzyme is part of naturally occurring biosynthetic pathways producing the branched chain amino acids, (i.e., valine, isoleucine, leucine), and pantothenic acid (vitamin B5). DHAD catalyzed conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate is also a common step in the multiple isobutanol biosynthetic pathways that are disclosed in U.S. Patent Appl. Pub. No. US 20070092957 A1, incorporated by reference herein. Disclosed therein is, *e.g.*, the engineering of recombinant microorganisms for the production of isobutanol.
- [0006] High levels of DHAD activity are desired for increased production of products from biosynthetic pathways that include this enzyme activity, including, *e.g.*, enhanced microbial production of branched chain amino acids, pantothenic acid, and isobutanol. Isobutanol, in particular, is useful as a fuel additive, and its ready availability may reduce the demand for petrochemical fuels. However, since all known DHAD enzymes require a Fe-S cluster for their function, they must be expressed in a host having the genetic machinery to provide the Fe-S clusters required by these proteins. In yeast, mitochondria play an essential role in Fe-S cluster biosynthesis. If the DHAD is to be functionally expressed in yeast cytosol, a system to transport the requisite Fe-S precursor or signal from mitochondria and assemble the Fe-S cluster on the cytosolic apoprotein is required. Prior to the work of the present inventors, it was previously unknown whether yeast could provide Fe-S clusters for any DHAD located in the cytoplasm (since native yeast DHAD is located in the mitochondria) and more importantly when the DHAD is expressed at high levels in the cytoplasm
- [0007] Under certain conditions the rate of synthesis of Fe-S cluster requiring apoproteins may exceed the cell's ability to synthesize and assemble Fe-S clusters for them. Cluster-less apo-proteins that accumulate under these conditions cannot carry out their normal function. Such conditions can include 1) the expression of a heterologous Fe-S cluster requiring protein especially in high amounts, 2) the expression of a native Fe-S cluster biosynthesis protein at higher levels than normal, or 3) a state where the host cell's ability to synthesize Fe-S clusters is debilitated.

BRIEF SUMMARY OF THE INVENTION

- [0008] Disclosed herein is the surprising discovery that recombinant host cells expressing a high level of a heterologous Fe-S cluster requiring protein can supply the complement of Fe-S clusters for that protein if the level(s) of at least one Fe uptake, utilization, and/or Fe-S cluster biosynthesis protein are altered.
- [0009] Provided herein are recombinant host cells comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity wherein said at least one heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated. Also provided are recombinant host cells comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity wherein said at least one heterologous polynucleotide is integrated at least once in the recombinant host cell DNA. Also provided are recombinant host cells comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity, wherein said host cell comprises at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting iron metabolism or Fe-S cluster Also provided are recombinant host cells comprising at least one biosynthesis. heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity and at least one heterologous polynucleotide encoding a polypeptide affecting iron metabolism or Fe-S cluster biosynthesis.
- [0010] In embodiments, said heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of the genes in Tables 7, 8 and 9. In embodiments, said heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, CCC1, FRA2, and GRX3, and combinations thereof. In embodiments, polypeptide is encoded by a polynucleotide that is constitutive mutant. In embodiments, said constitutive mutant is selected from the group consisting of AFT1 L102A, AFT1 C291F, AFT1 C293F, and combinations thereof. In embodiments said polypeptide affecting Fe-S cluster biosynthesis is encoded by a polynucleotide comprising a high copy number plasmid or a plasmid with a copy number that can be regulated. In embodiments, said polypeptide affecting Fe-S cluster biosynthesis is encoded by a polynucleotide integrated at least once in the recombinant host cell DNA.

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In embodiments, the at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of CCC1, FRA2, and GRX3, and combinations thereof. In embodiments, the at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, their mutants, and combinations thereof.

- [0011] In embodiments, said at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is expressed in multiple copies. In embodiments, said at least one heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated. In embodiments, said at least one heterologous polynucleotide is integrated at least once in the recombinant host cell DNA. In embodiments, said Fe-S cluster biosynthesis is increased compared to a recombinant host cell having endogenous Fe-S cluster biosynthesis.
- [0012] In embodiments, said host cell is a yeast host cell. In embodiments, said yeast host cell is selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia* and *Pichia*.
- [0013] In embodiments, said heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in the cytosol of the host cell. In embodiments, said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of $< 10^{-5}$ wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the Streptococcus mutans DHAD enzyme corresponding to SEQ ID NO:168. In embodiments, said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence with at least about 90% identity to SEQ ID NO: 168 or SEQ ID NO: 232. In embodiments said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of: greater than about 5-fold with respect to the control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity, greater than about 8-fold with respect to the control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity, or greater than about 10-fold with respect to the control host cell comprising at least one heterologous

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polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity. In embodiments said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of: greater than about 3-fold with respect to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity and greater than about 6-fold with respect to the control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity. In embodiments, said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of: greater than about 0.25 U/mg; greater than about 0.3 U/mg; greater than about 0.5 U/mg; greater than about 1.0 U/mg; greater than about 2.0 U/mg; greater than about 3.0 U/mg; greater than about 5.0 U/mg; greater than about 4.0 U/mg; greater than about 5.0 U/mg; greater than about 5.0 U/mg; greater than about 10.0 U/mg; greater than about 2.0 U/mg; greater than about 5.0 U/mg; greater than about 1.0 U/mg; greater than about 1.0 U/mg; greater than about 2.0 U/mg; greater than about 5.0 U/mg; greater than about 1.0 U/mg; greater than about 5.0 U/mg; greater than

- [0014] In embodiments said recombinant host cell produces isobutanol, and in embodiments, said recombinant host cell comprises an isobutanol biosynthetic pathway.
- [0015] Also provided herein are methods of making a product comprising: providing a recombinant host cell; and contacting the recombinant host cell of with a fermentable carbon substrate in a fermentation medium under conditions wherein said product is produced;, wherein the product is selected from the group consisting of branched chain amino acids, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol, and combinations thereof. In embodiments, the methods further comprise optionally recovering said product.
- [0016] Also provided are methods of making isobutanol comprising: providing a recombinant host cell; contacting the recombinant host cell with a fermentable carbon substrate in a fermentation medium under conditions wherein isobutanol is produced. In embodiments, the methods further comprise optionally recovering said isobutanol. In embodiments, the methods further comprise recovering said isobutanol.
- [0017] Also provided are methods for the conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate comprising: providing a recombinant host cell; growing the recombinant

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host cell of under conditions where the 2,3-dihydroxyisovalerate is converted to α ketoisovalerate. In embodiments, the conversion of 2,3-dihydroxyisovalerate to α ketoisovalerate compared to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is increased in an amount selected from the group consisting of: (a) at least about 5%; (b) at least about 10%; (c) at least about 15%; (d) at least about 20%; (e) at least about 25%; (f) at least about 30%; (g) at least about 35%; (h) at least about 40%; (i) at least about 45%; (j) at least about 50%; (k) at least about 60%; (l) at least about 70%; (m) at least about 80%; (n) at least about 90%; and (o) at least about 95%.

- [0018] Also provided are methods for increasing the specific activity of a heterologous polypeptide having dihydroxy-acid dehydratase activity in a recombinant host cell comprising: providing a recombinant host cell; and growing the recombinant host cell of under conditions whereby the heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in functional form having a specific activity greater than the same host cell lacking said heterologous polypeptide.
- [0019] Also provided are methods for increasing the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising: providing a recombinant host cell; and growing the recombinant host cell under conditions whereby the flux in the Fe-S cluster biosynthesis pathway in the host cell is increased.
- [0020] Also provide are methods of increasing the activity of an Fe-S cluster requiring protein in a recombinant host cell comprising: providing a recombinant host cell comprising an Fe-S cluster requiring protein; changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis in said host cell; and growing the recombinant host cell under conditions whereby the activity of the Fe-S cluster requiring protein is increased. In embodiments, said increase in activity is an amount selected from the group consisting of: greater than about 10%; greater than about 20%; greater than about 30%; greater than about 40%; greater than about 50%; greater than about 60%; greater than about 70%; greater than about 80%; greater than about 90%; and greater than about 95%, 98%, or 99%. In embodiments, the increase in activity is in an amount selected from the group consisting of: greater than about 5-fold; greater than about 8-fold; greater than about 10-fold. In embodiments, the increase in activity is in an amount

selected from the group consisting of: greater than about 3-fold and greater than about 6-fold.

- [0021] A method for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising: changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis; measuring the activity of a heterologous Fe-S cluster requiring protein; and comparing the activity of the heterologous Fe-S cluster requiring protein measured in the presence of the change in expression or activity of a polypeptide to the activity of the heterologous Fe-S cluster requiring protein measured in expression or activity of a polypeptide, wherein an increase in the activity of the heterologous Fe-S cluster requiring protein an increase in the activity of the heterologous Fe-S cluster requiring protein an increase in the flux in said Fe-S cluster biosynthesis pathway.
- **[0022]** Provided herein are methods for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising: changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis; measuring the activity of a polypeptide having dihydroxy-acid dehydratase activity; and comparing the activity of the polypeptide having dihydroxy-acid dehydratase activity measured in the presence of the change to the activity of the polypeptide having dihydroxy-acid dehydratase in the activity of the polypeptide having dihydroxy-acid dehydratase activity measured in the absence of change, wherein an increase in the activity of the polypeptide having dihydroxy-acid dehydratase activity indicates an increase in the flux in said Fe-S cluster biosynthesis pathway.
- [0023] In embodiments, said changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis comprises deleting, mutating, substituting, expressing, up-regulating, down-regulating, altering the cellular location, altering the state of the protein, and/or adding a cofactor. In embodiments, the Fe-S cluster requiring protein has dihydroxy-acid dehydratase activity and wherein said Fe-S cluster requiring protein having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of $< 10^{-5}$ wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the *Streptococcus mutans* DHAD enzyme corresponding to SEQ ID NO:168. In embodiments, the polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of the genes in Tables 7, 8 and 9.

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- [0024] Also provided are recombinant host cells comprising at least one polynucleotide encoding a polypeptide identified by the methods provided herein. In embodiments, said host cell further comprises at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity. In embodiments, said heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is expressed in multiple copies. In embodiments, said heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated. In embodiments, said heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.
- In embodiments, said host cell is a yeast host cell. In embodiments, said yeast [0025] host cell is selected from the group consisting of Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia, and Pichia. In embodiments, said heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in the cytosol of the host cell. In embodiments, said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of $< 10^{-5}$ wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the Streptococcus mutans DHAD enzyme corresponding to SEQ ID NO:168. In embodiments, said recombinant host cell produces a product selected from the group consisting of branched chain amino acids, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol, and combinations thereof. In embodiments, recombinant host cell produces isobutanol. In embodiments, said recombinant host cell comprises an isobutanol biosynthetic pathway. In embodiments said isobutanol biosynthetic pathway comprises at least one polypeptide encoded by a polynucleotide heterologous to the host cell. In embodiments, said isobutanol biosynthetic pathway comprises at least two polypeptides encoded by polynucleotides heterologous to the host cell.
- [0026] In embodiments, monomers of the polypeptides of the invention having dihydroxy-acid dehydratase activity have an Fe-S cluster loading selected from the group consisting of: (a) at least about 10%; (b) at least about 15%; (c) at least about 20%; (d) at least about 25%; (e) at least about 30%; (f) at least about 35%; (g) at least about 40%; (h)

at least about 45%; (i) at least about 50%; (j) at least about 60%; (k) at least about 70%; (l) at least about 80%; (m) at least about 90%; and (n) at least about 95%.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

- [0027] Figure 1A depicts a vector map of a vector for overexpression of the *IlvD* gene from *S. mutans*.
- [0028] Figure 1B depicts a vector map of an integration vector for overexpression of the *IlvD* gene from *S. mutans* in the chromosome.
- [0029] Figure 2 depicts a vector map of a centromere vector used to clone *AFT1* or *AFT1* mutants and useful for other genes of interest.
- [0030] Figure 3 depicts a UV-Vis absorbance spectrum of purified *S. mutans* DHAD.
- [0031] Figure 4 depicts an EPR spectrum of purified *S. mutans* DHAD.
- [0032] Figure 5 depicts a biosynthetic pathway for biosynthesis of isobutanol.
- [0033] Figure 6A depicts a schematic of *Azotobacter vinelandii nif* genes.
- [0034] Figure 6B depicts a schematic of additional *Azotobacter vinelandii nif* genes.
- [0035] Figure 6C depicts a schematic of the equation in which NFU acts as a persulfide reductase.
- [0036] Figure 7 depicts a schematic of *Helicobacter pylori nif* genes.
- [0037] Figure 8 depicts a schematic of *E. coli isc* genes.
- [0038] Figure 9 depicts a schematic of *E. coli suf* genes.
- [0039] Figure 10 depicts a schematic of the cytosolic [2Fe-2S] biosynthesis and assembly system.
- [0040] Figure 11 depicts a vector map of a vector for overexpression of the *IlvD* gene from *L. lactis*.
- [0041] Table 12 is a table of the Profile HMM for dihydroxy-acid dehydratases based on enzymes with assayed function prepared as described in U.S. Patent Appl. No. 12/569,636, filed Sept. 29, 2009. Table 12 is submitted herewith electronically and is incorporated herein by reference.

DETAILED DESCRIPTION OF THE INVENTION

[0042] Described herein is a method to increase the fraction of the Fe-S cluster requiring proteins that are loaded with Fe-S clusters. Also described are recombinant host cells that express functional Fe-S cluster requiring proteins, such as DHAD enzymes, and at least one heterologous Fe uptake, utilization, or Fe-S cluster biosynthesis protein, recombinant host cells that express functional DHAD enzymes and comprise at least one deletion, mutation, and/or substitution in a native protein involved in Fe utilization or Fe-S cluster biosynthesis, or recombinant host cells comprising combinations thereof. In addition, the present invention describes a method to identify polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell. Also described is a method to identify polypeptides that alter the activity of an Fe-S cluster requiring protein.

[0043] <u>Definitions</u>

- [0044] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present application including the definitions will control. Also, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. All publications, patents and other references mentioned herein are incorporated by reference in their entireties for all purposes.
- [0045] In order to further define this invention, the following terms and definitions are herein provided.
- [0046] As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains" or "containing," or any other variation thereof, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. For example, a composition, a mixture, a process, a method, an article, or an apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

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- [0047] As used herein, the term "consists of," or variations such as "consist of" or "consisting of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, but that no additional integer or group of integers may be added to the specified method, structure, or composition.
- [0048] As used herein, the term "consists essentially of," or variations such as "consist essentially of" or "consisting essentially of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, and the optional inclusion of any recited integer or group of integers that do not materially change the basic or novel properties of the specified method, structure or composition. *See* M.P.E.P. § 2111.03.
- [0049] Also, the indefinite articles "a" and "an" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances, *i.e.*, occurrences of the element or component. Therefore "a" or "an" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.
- [0050] The term "invention" or "present invention" as used herein is a non-limiting term and is not intended to refer to any single embodiment of the particular invention but encompasses all possible embodiments as described in the application.
- [0051] As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or to carry out the methods; and the like. The term "about" also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term "about" means within 10% of the reported numerical value, preferably within 5% of the reported numerical value.
- [0052] The term "isobutanol biosynthetic pathway" refers to an enzyme pathway to produce isobutanol from pyruvate.

- [0053] The term "a facultative anaerobe" refers to a microorganism that can grow in both aerobic and anaerobic environments.
- [0054] The term "carbon substrate" or "fermentable carbon substrate" refers to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof.
- [0055] The term "Fe-S cluster biosynthesis" refers to biosynthesis of Fe-S clusters, including, *e.g.*, the assembly and loading of Fe-S clusters. The term "Fe-S cluster biosynthesis genes", "Fe-S cluster biosynthesis proteins" or "Fe-S cluster biosynthesis pathway" refers to those polynucleotides/genes and the encoded polypeptides that are involved in the biosynthesis of Fe-S clusters, including, *e.g.*, the assembly and loading of Fe-S clusters.
- [0056] The term "Fe uptake and utilization" refers to processes which can effect Fe-S cluster biosynthesis such as Fe sensing, uptake, utilization, and homeostasis. "Fe uptake and utilization genes" refers to those polynucleotides/genes and the encoded polypeptides that are involved in Fe uptake, utilization, and homeostasis. Some of these polynucleotides/genes are contained in the "Fe Regulon" that has been described in the literature and is further described hereafter. As used herein, Fe uptake and utilization genes and Fe-S cluster biosynthesis genes can encode a polypeptide affecting Fe-S cluster biosynthesis.
- [0057] The term "specific activity" as used herein is defined as the units of activity in a given amount of protein. Thus, the specific activity is not directly measured but is calculated by dividing 1) the activity in units/ml of the enzyme sample by 2) the concentration of protein in that sample, so the specific activity is expressed as units/mg. The specific activity of a sample of pure, fully active enzyme is a characteristic of that enzyme. The specific activity of a sample of a mixture of proteins is a measure of the relative fraction of protein in that sample that is composed of the active enzyme of interest. The specific activity of a polypeptide of the invention may be selected from greater than about 0.25 U/mg; greater than about 0.3 U/mg; greater than about 0.4 U/mg; greater than about 0.5 U/mg; greater than about 0.9 U/mg; greater than about 1.0 U/mg; greater than about 1.5 U/mg; greater than about 2.0 U/mg; greater than about 2.5 U/mg;

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greater than about 3.0 U/mg; greater than about 3.5 U/mg; greater than about 4.0 U/mg; greater than about 5.5 U/mg; greater than about 5.0 U/mg; greater than about 6.0 U/mg; greater than about 6.5 U/mg; greater than about 7.0 U/mg; greater than about 7.5 U/mg; greater than about 8.0 U/mg; greater than about 8.5 U/mg; greater than about 9.0 U/mg; greater than about 9.5 U/mg; greater than about 10.0 U/mg; greater than about 20.0 U/mg; or greater than about 50.0 U/mg. In one embodiment, the specific activity of a polypeptide of the invention is greater than about 0.25 U/mg. In another embodiment, the specific activity is greater than about 1.0 U/mg. In yet another embodiment, the specific activity is greater than about 2.0 U/mg or greater than about 3.0 U/mg.

- **[0058]** The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to a nucleic acid molecule or construct, *e.g.*, messeger RNA (mRNA) or plasmid DNA (pDNA). A polynucleotide can contain the nucleotide sequence of the full-length cDNA sequence, or a fragment thereof, including the untranslated 5' and 3' sequences and the coding sequences. The polynucleotide can be composed of any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. "Polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.
- [0059] A polynucleotide sequence may be referred to as "isolated," in which it has been removed from its native environment. For example, a heterologous polynucleotide encoding a polypeptide or polypeptide fragment having dihydroxy-acid dehydratase activity contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. An isolated polynucleotide fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

- **[0060]** The term "gene" refers to a polynucleotide that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature.
- [0061] As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. Two or more coding regions of the present invention can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions. In addition, a vector, polynucleotide, or nucleic acid of the invention may encode heterologous coding regions.
- [0062] The term "endogenous," when used in reference to a polynucleotide, a gene, or a polypeptide refers to a native polynucleotide or gene in its natural location in the genome of an organism, or for a native polypeptide, is transcribed and translated from this location in the genome.
- **[0063]** The term "heterologous" when used in reference to a polynucleotide, a gene, or a polypeptide refers to a polynucleotide, gene, or polypeptide not normally found in the host organism. "Heterologous" also includes a native coding region, or portion thereof, that is reintroduced into the source organism in a form that is different from the corresponding native gene, *e.g.*, not in its natural location in the organism's genome. The heterologous polynucleotide or gene may be introduced into the host organism by, *e.g.*, gene transfer. A heterologous gene may include a native coding region with non-native regulatory regions that is reintroduced into the native host. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

- [0064] The term "recombinant genetic expression element" refers to a nucleic acid fragment that expresses one or more specific proteins, including regulatory sequences preceding (5' non-coding sequences) and following (3' termination sequences) coding sequences for the proteins. A chimeric gene is a recombinant genetic expression element. The coding regions of an operon may form a recombinant genetic expression element, along with an operably linked promoter and termination region.
- [0065] "Regulatory sequences" refers to nucleotide sequences located upstream (5' noncoding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, enhancers, operators, repressors, transcription termination signals, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.
- [0066] The term "promoter" refers to a nucleic acid sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleic acid segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". "Inducible promoters," on the other hand, cause a gene to be expressed when the promoter is induced or turned on by a promoter-specific signal or molecule. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.
- [0067] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of effecting the expression of that coding sequence (*i.e.*, that the coding sequence is under

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the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

- [0068] The term "expression", as used herein, refers to the transcription and accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. The process includes any manifestation of the functional presence of the expressed polynucleotide, gene, or polypeptide within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression.
- **[0069]** The term "over-expression", as used herein, refers to expression that is higher than endogenous expression of the same or related polynucleotide or gene. A heterologous polynucleotide or gene is also over-expressed if its expression is higher than that of a comparable endogenous gene, or if its expression is higher than that of the same polynucleotide or gene introduced by a means that does not overexpress the polynucleotide or gene. For example, a polynucleotide can be expressed in a host cell from a low copy number plasmid, which is present in only limited or few copies, and the same polynucleotide can be over-expressed in a host cell from a high copy number plasmid or a plasmid with a copy number that can be regulated, which is present in multiple copies. Any means can be used to over-express a polynucleotide, so long as it increases the copies of the polynucleotide in the host cell. In addition to using a high copy number plasmid, or a plasmid with a copy number that can be regulated, a polynucleotide can be over-expressed by multiple chromosomal integrations.
- [0070] Expression or over-expression of a polypeptide of the invention in a recombinant host cell can be quantified according to any number of methods known to the skilled artisan and can be represented, *e.g.*, by a percent of total cell protein. The percent of total protein can be an amount selected from greater than about 0.001% of total cell protein; greater than about 0.01% of total cell protein; greater than about 0.01% of total cell protein; greater than about 0.5% of total cell protein; greater than about 1.0% of total cell protein; greater than about 2.0% of total cell protein; greater than about 3% of total cell protein; greater than about 4.0% of total cell protein; greater than about 5% of total cell protein; greater than about 4.0% of total cell protein; greater than about 5% of total cell protein; greater than about 5.0% of total cell protein; greater than

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cell protein. In one embodiment, the amount of polypeptide expressed is greater that about 0.5% of total cell protein. In another embodiment, the amount of polypeptide expressed is greater than about 1.0% of total cell protein or greater than about 2.0% of total cell protein.

- [0071] As used herein the term "transformation" refers to the transfer of a nucleic acid fragment into a host organism, resulting in genetically stable inheritance with or without selections. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.
- **[0072]** The terms "plasmid" and "vector" as used herein, refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.
- [0073] As used herein the term "codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.
- [0074] The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that organism.

[0075] Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). The "genetic code" which shows which codons encode which amino acids is reproduced herein as Table 1. As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

Γ	С	Α	G

Table 1. The Standard Genetic Code

	Т	С	Α	G
Т	TTC "	TCT Ser (S) TCC " TCA " TCG "	TAT Tyr (Y) TAC " TAA Stop TAG Stop	TGT Cys (C) TGC TGA Stop TGG Trp (W)
С	CTT Leu (L) CTC " CTA " CTG "	CCT Pro (P) CCC " CCA " CCG "	CAT His (H) CAC " CAA Gln (Q) CAG "	CGT Arg (R) CGC " CGA " CGG "
Α	ATT Ile (I) ATC " ATA " ATG Met (M)	ACC "	AAC "	AGT Ser (S) AGC " AGA Arg (R) AGG "
G	GTT Val (V) GTC " GTA " GTG "	GCT Ala (A) GCC " GCA " GCG "	GAT Asp (D) GAC " GAA Glu (E) GAG "	GGT Gly (G) GGC " GGA " GGG "

[0076] Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference, or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic

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code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, *inter alia*, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

- [0077]
- Given the large number of gene sequences available for a wide variety of animal, plant and microbial species, it is possible to calculate the relative frequencies of codon usage. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at http://www.kazusa.or.jp/codon/ (visited March 20, 2008), and these tables can be adapted in a number of ways. See Nakamura, Y., et al. Nucl. Acids Res. 28:292 (2000). Codon usage tables for yeast, calculated from GenBank Release 128.0 [15 February 2002], are reproduced below as Table 2. This table uses mRNA nomenclature, and so instead of thymine (T) which is found in DNA, the tables use uracil (U) which is found in RNA. Table 2 has been adapted so that frequencies are calculated for each amino acid, rather than for all 64 codons.

Amino Acid	Codon	Number	Frequency per thousand
Phe	UUU	170666	26.1
Phe	UUC	120510	18.4
Leu	UUA	170884	26.2
Leu	UUG	177573	27.2
Leu	CUU	80076	12.3
Leu	CUC	35545	5.4
Leu	CUA	87619	13.4
Leu	CUG	68494	10.5
Ile	AUU	196893	30.1
Ile	AUC	112176	17.2
Ile	AUA	116254	17.8
Met	AUG	136805	20.9
Val	GUU	144243	22.1

 Table 2. Codon Usage Table for Saccharomyces cerevisiae Genes

Val GUC 76947 11.8 Val GUA 76927 11.8 Val GUG 70337 10.8 Ser UCC 92923 14.2 Ser UCA 122028 18.7 Ser UCG 55951 8.6 Ser AGU 92466 14.2 Ser AGC 63726 9.8 Pro CCU 88263 13.5 Pro CCC 44309 6.8 Pro CCG 34597 5.3 Thr ACU 132522 20.3 Thr ACC 83207 12.7 Thr ACG 52045 8.0 Ala GCC 82357 12.6 Ala GCC 82357 12.6 Ala GCA 105910 16.2 Ala GCG 40358 6.2 Tyr UAU 122728	Amino Acid	Codon	Number	Frequency per thousand
Val GUA 76927 11.8 Val GUG 70337 10.8 Ser UCU 153557 23.5 Ser UCC 92923 14.2 Ser UCA 122028 18.7 Ser UCG 55951 8.6 Ser AGU 92466 14.2 Ser AGC 63726 9.8 Pro CCU 88263 13.5 Pro CCC 44309 6.8 Pro CCA 119641 18.3 Pro CCG 34597 5.3 Thr ACU 132522 20.3 Thr ACU 132522 20.3 Thr ACC 83207 12.7 Thr ACG 52045 8.0 Ala GCC 82357 12.6 Ala GCC 82357 12.6 Ala GCG 40358 6.2 Tyr UAU 122728 18.8 <td>Val</td> <td>GUC</td> <td>76947</td> <td></td>	Val	GUC	76947	
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	Lys	AAG	201361	30.8
Asp GAU 245641 37.6	Asn	GAU	245641	37.6
Asp GAU 245641 37.6 Asp GAC 132048 20.2				

Amino Acid	Codon	Number	Frequency per thousand
Glu	GAA	297944	45.6
Glu	GAG	125717	19.2
Cys	UGU	52903	8.1
Cys	UGC	31095	4.8
	_		
Trp	UGG	67789	10.4
	-		
Arg	CGU	41791	6.4
Arg	CGC	16993	2.6
Arg	CGA	19562	3.0
Arg	CGG	11351	1.7
Arg	AGA	139081	21.3
Arg	AGG	60289	9.2
Gly	GGU	156109	23.9
Gly	GGC	63903	9.8
Gly	GGA	71216	10.9
Gly	GGG	39359	6.0
Stop	UAA	6913	1.1
Stop	UAG	3312	0.5
Stop	UGA	4447	0.7

- [0078] By utilizing this or similar tables, one of ordinary skill in the art can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide, but which uses codons optimal for a given species.
- [0079] Randomly assigning codons at an optimized frequency to encode a given polypeptide sequence, can be done manually by calculating codon frequencies for each amino acid, and then assigning the codons to the polypeptide sequence randomly. Additionally, various algorithms and computer software programs are readily available to those of ordinary skill in the art. For example, the "EditSeq" function in the Lasergene Package, available from DNAstar, Inc., Madison, WI, the backtranslation function in the VectorNTI Suite, available from InforMax, Inc., Bethesda, MD, and the "backtranslate" function in the GCG-Wisconsin Package, available from Accelrys, Inc., San Diego, CA. In addition, various resources are publicly available to codon-optimize coding region sequences, the "backtranslation" function e.g., at

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http://www.entelechon.com/bioinformatics/backtranslation.php?lang=eng (visited April 15, 2008) and the "backtranseq" function available at http://bioinfo.pbi.nrc.ca:8090/EMBOSS/index.html (visited July 9, 2002). Constructing a rudimentary algorithm to assign codons based on a given frequency can also easily be accomplished with basic mathematical functions by one of ordinary skill in the art.

- [0080] Codon-optimized coding regions can be designed by various methods known to those skilled in the art including software packages such as "synthetic gene designer" (http://phenotype.biosci.umbc.edu/codon/sgd/index.php).
- [0081] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein, " "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis.
- [0082] By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purposed of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.
- [0083] As used herein, the term "variant" refers to a polypeptide differing from a specifically recited polypeptide of the invention, such as DHAD, by amino acid insertions, deletions, mutations, and substitutions, created using, *e.g.*, recombinant DNA techniques, such as mutagenesis. Guidance in determining which amino acid residues may be replaced, added, or deleted without abolishing activities of interest, may be found by comparing the sequence of the particular polypeptide with that of homologous

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polypeptides, *e.g.*, yeast or bacterial, and minimizing the number of amino acid sequence changes made in regions of high homology (conserved regions) or by replacing amino acids with consensus sequences.

- **[0084]** Alternatively, recombinant polynucleotide variants encoding these same or similar polypeptides may be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector for expression. Mutations in the polynucleotide sequence may be reflected in the polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide. For example, mutations can be used to reduce or eliminate expression of a target protein and include, but are not limited to, deletion of the entire gene or a portion of the gene, inserting a DNA fragment into the gene (in either the promoter or coding region) so that the protein is not expressed or expressed at lower levels, introducing a mutation into the coding region which adds a stop codon or frame shift such that a functional protein is not expressed, and introducing one or more mutations into the coding region to alter amino acids so that a non-functional or a less enzymatically active protein is expressed.
- [0085] Amino acid "substitutions" may be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, *i.e.*, conservative amino acid replacements, or they may be the result of replacing one amino acid with an amino acid having different structural and/or chemical properties, *i.e.*, non-conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Alternatively, "non-conservative" amino acid substitutions may be made by selecting the differences in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of any of these amino acids. "Insertions" or "deletions" may be within the range of variation as structurally or

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functionally tolerated by the recombinant proteins. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

- [0086] A "substantial portion" of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Altschul, S. F., et al., J. Mol. Biol., 215:403-410 (1993)). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., in situ hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence. The instant specification teaches the complete amino acid and nucleotide sequence encoding particular proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.
- [0087] The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenine is complementary to thymine and cytosine is complementary to guanine, and with respect to RNA, adenine is complementary to uracil and cytosine is complementary to guanine.

- [0088] The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: 1.) Computational Molecular Biology (Lesk, A. M., Ed.) Oxford University: NY (1988); 2.) Biocomputing: Informatics and Genome Projects (Smith, D. W., Ed.) Academic: NY (1993); 3.) Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., Eds.) Humania: NJ (1994); 4.) Sequence Analysis in Molecular Biology (von Heinje, G., Ed.) Academic (1987); and 5.) Sequence Analysis Primer (Gribskov, M. and Devereux, J., Eds.) Stockton: NY (1991).
- [0089] Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the MegAlign[™] program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignments of the sequences is performed using the "Clustal method of alignment" which encompasses several varieties of the algorithm including the "Clustal V method of alignment" corresponding to the alignment method labeled Clustal V (described by Higgins and Sharp, CABIOS. 5:151-153 (1989); Higgins, D.G. et al., Comput. Appl. Biosci., 8:189-191 (1992)) and found in the MegAlign[™] program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program. Additionally the "Clustal W method of alignment" is available and corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, CABIOS. 5:151-153 (1989); Higgins, D.G. et

al., Comput. Appl. Biosci. 8:189-191(1992)) and found in the MegAlign[™] v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

- [0090]
- 90] It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, from other species, wherein such polypeptides have the same or similar function or activity, or in describing the corresponding polynucleotides. Useful examples of percent identities include, but are not limited to: 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from 55% to 100% may be useful in describing the present invention, such as 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. Suitable polynucleotide fragments not only have the above homologies but typically comprise a polynucleotide having at least 50 nucleotides. Further, suitable polynucleotide fragments having the above homologies encode a polypeptide having at least 50 amino acids, at least 100 amino acids, at least 150 amino acids, at least 200 amino acids.
- [0091] The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to: 1.) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI); 2.) BLASTP, BLASTN, BLASTX (Altschul et al., J. Mol. Biol., 3.) DNASTAR 215:403-410 (1990)); (DNASTAR, Inc. Madison. WI): 4.) SEQUENCHER (Gene Codes Corporation, Ann Arbor, MI); and 5.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, Comput. Methods Genome Res., [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor.

Plenum: New York, NY). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

- [0092] Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1984); and by Ausubel, F. M. *et al.*, *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience (1987).
- [0093] <u>The Functions of Fe-S Cluster-Requiring Proteins</u>
- [0094] The functions of proteins that contain Fe-S clusters are diverse. One of the more complete efforts to classify these functions is given in the following table which is adapted from Johnson, D.C., et al., *Structure, function, and formation of biological iron-sulfur clusters*. Annu. Rev. Biochem., 2005. 74: p. 247-281.

Function	Examples	Cluster type
	Examples	Cluster type
Electron transfer	Ferredoxins; redox	[2Fe-2S]; [3Fe-4S]; [4Fe-4S]
	enzymes	
Coupled electron/proton	Rieske protein	[2Fe-2S]
transfer	Nitrogenase	[8Fe-7S]
Substrate binding and	(de)Hydratases	[4Fe-4S], [2Fe-2S]
activation	Radical SAM enzymes	[4Fe-4S]
	Acetyl-CoA synthase	Ni-Ni-[4Fe-4S], [Ni-4Fe-5S]
	Sulfite reductase	[4Fe-4S]-siroheme
Fe or cluster storage	Ferredoxins	[4Fe-4S]
	Polyferredoxins	[4Fe-4S]
Structural	Endonuclease III	[4Fe-4S]
	MutY	[4Fe-4S]
Regulation of gene expression	SoxR	[2Fe-2S]
	FNR	[4Fe-4S]/[2Fe-2S]
	IRP	[4Fe-4S]
	IscR	[2Fe-2S]
Regulation of enzyme activity	Glutamine PRPP	[4Fe-4S]
	amidotransferase	
	Ferrochelatase	[2Fe-2S]
Disulfide reduction	Ferredoxin:thioredoxin	[4Fe-4S]
	reductase	
	Heterodisulfide reductase	[4Fe-4S]
Sulfur donor	Biotin synthase	[2Fe-2S]

Table 3. Functions of Biological [Fe-S] clusters^a.

^aAbbreviations used are SAM, S-adenosylmethionine; acetyl-CoA, acetyl coenzymeA; FNR, fumarate and nitrate reduction; IRP, iron-regulatory protein; IscR, iron-sulfur cluster assembly regulatory protein; PRPP, phosphoribosylpyrophosphate.

[0095] It is believed that an increase in the supply and the efficiency of loading Fe-S clusters into one or more of the members of the above classes will have commercial and/or medical benefits. Of the many possibilities that will be appreciated by the skilled artisan, three examples are given. 1) When an Fe-S cluster containing enzyme is used in a pathway to a fermentation product and needs to be expressed at high levels to maintain a high flux in the pathway to the product (e.g., dihydroxy-acid dehydratase in the pathway to isobutanol). 2) When an Fe-S cluster containing enzyme is used in a pathway to a fermentation product and the Fe-S cluster undergoes turnover during the catalysis (e.g., biotin synthase in the commercial fermentation of glucose to biotin). 3) In a diseased state such that the normal concentration of an Fe-S cluster containing protein important for good health is low (e.g., in cases of Friedreich's ataxia).

[0096] DHAD and DHAD Assays

[0097] DHAD is an Fe-S cluster requiring protein of the dehydratase (more properly hydro-lyase) class. A gene encoding a DHAD enzyme can be used to provide expression of DHAD activity in a recombinant host cell. DHAD catalyzes the conversion of 2,3dihydroxyisovalerate to α -ketoisovalerate and of 2,3-dihydroxymethylvalerate to α ketomethylvalerate and is classified as E.C. 4.2.1.9. Coding sequences for DHADs that are suitable for use in a recombinant host cell can be derived from bacterial, fungal, or plant sources. DHADs that may be used may have a [4Fe-4S] cluster or a [2Fe-2S]. Tables 4a, 4b, 5, and 6 list SEQ ID NOs for coding regions and proteins of representative DHADs that may be used in the present invention. Proteins with at least about 95% identity to certain listed sequences have been omitted for simplification, but it is understood that proteins, including those omitted for simplification, with at least about 95% sequence identity to any of the proteins listed in Tables 4a, 4b, 5, and 6 and having DHAD activity may be used as disclosed herein. Additional DHAD proteins and their encoding sequences may be identified by BLAST searching of public databases, as well known to one skilled in the art. Typically BLAST (described above) searching of publicly available databases with known DHAD sequences, such as those provided herein, is used to identify DHADs and their encoding sequences that may be expressed in the present cells. For example, DHAD proteins having amino acid sequence identities of at least about 80-85%, at least about 85-90%, at least about 90-95%, or at least about 98% sequence identity to any of the DHAD proteins of Table 3 may be expressed in the present cells. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

Table 4a. SEQ ID NOs of Representative Bacterial [2Fe-2S] DHAD Proteins and Encoding Sequences.

Organism of derivation	SEQ ID NO:	SEQ ID NO:
	Nucleic acid	Peptide
Mycobacterium sp. MCS	1	2
Mycobacterium gilvum PYR-GCK	3	4
Mycobacterium smegmatis str. MC2 155	5	6
Mycobacterium vanbaalenii PYR-1	7	8
Nocardia farcinica IFM 10152	9	10

Rhodococcus sp. RHA1	11	12
Mycobacterium ulcerans Agy99	13	14
Mycobacterium avium subsp. paratuberculosis K-10	15	16
Mycobacterium tuberculosis H37Ra	17	18
Mycobacterium leprae TN *	19	20
Kineococcus radiotolerans SRS30216	21	22
Janibacter sp. HTCC2649	23	24
Nocardioides sp. JS614	25	26
Renibacterium salmoninarum ATCC 33209	27	28
Arthrobacter aurescens TC1	29	30
Leifsonia xyli subsp. xyli str. CTCB07	31	32
marine actinobacterium PHSC20C1	33	34
Clavibacter michiganensis subsp. michiganensis	35	36
NCPPB 382	33	50
Saccharopolyspora erythraea NRRL 2338	37	38
Acidothermus cellulolyticus 11B	39	40
Corynebacterium efficiens YS-314	41	42
Brevibacterium linens BL2	43	44
Tropheryma whipplei TW08/27	45	46
Methylobacterium extorquens PA1	47	48
Methylobacterium nodulans ORS 2060	49	50
Rhodopseudomonas palustris BisB5	51	52
Rhodopseudomonas palustris BisB18	53	54
Bradyrhizobium sp. ORS278	55	56
Bradyrhizobium japonicum USDA 110	57	58
Fulvimarina pelagi HTCC2506	59	60
Aurantimonas sp. SI85-9A1	61	62
Hoeflea phototrophica DFL-43	63	64
Mesorhizobium loti MAFF303099	65	66
Mesorhizobium sp. BNC1	67	68
Parvibaculum lavamentivorans DS-1	69	70
Loktanella vestfoldensis SKA53	71	72
Roseobacter sp. CCS2	73	74
Dinoroseobacter shibae DFL 12	75	76
Roseovarius nubinhibens ISM	77	78
Sagittula stellata E-37	79	80
Roseobacter sp. AzwK-3b	81	82
Roseovarius sp. TM1035	83	84

Oceanicola batsensis HTCC2597	85	86
Oceanicola granulosus HTCC2516	87	88
Rhodobacterales bacterium HTCC2150	89	90
Paracoccus denitrificans PD1222	91	92
Oceanibulbus indolifex HEL-45	93	94
Sulfitobacter sp. EE-36	95	96
Roseobacter denitrificans OCh 114	97	98
Jannaschia sp. CCS1	99	100
Caulobacter sp. K31	101	102
Candidatus Pelagibacter ubique HTCC1062	103	104
Erythrobacter litoralis HTCC2594	105	106
Erythrobacter sp. NAP1	107	108
Comamonas testosterone KF-1	109	110
Sphingomonas wittichii RW1	111	112
Burkholderia xenovorans LB400	113	114
Burkholderia phytofirmans PsJN	115	116
Bordetella petrii DSM 12804	117	118
Bordetella bronchiseptica RB50	119	120
Bradyrhizobium sp. ORS278	121	122
Bradyrhizobium sp. BTAil	123	124
Bradhyrhizobium japonicum	125	126
Sphingomonas wittichii RW1	127	128
Rhodobacterales bacterium HTCC2654	129	130
Solibacter usitatus Ellin6076	131	132
Roseiflexus sp. RS-1	133	134
Rubrobacter xylanophilus DSM 9941	135	136
Salinispora tropica CNB-440	137	138
Acidobacteria bacterium Ellin345	139	140
Thermus thermophilus HB27	141	142
Maricaulis maris MCS10	143	144
Parvularcula bermudensis HTCC2503	145	146
Oceanicaulis alexandrii HTCC2633	147	148
Plesiocystis pacifica SIR-1	149	150
Bacillus sp. NRRL B-14911	151	152
Oceanobacillus iheyensis HTE831	153	154
Staphylococcus saprophyticus subsp. saprophyticus	155	156
ATCC 15305	155	150
Bacillus selenitireducens MLS10	157	158

Streptococcus pneumoniae SP6-BS73	159	160
Streptococcus sanguinis SK36	161	162
Streptococcus thermophilus LMG 18311	163	164
Streptococcus suis 89/1591	165	166
Streptococcus mutans UA159	167	168
Leptospira borgpetersenii serovar Hardjo-bovis L550	169	170
Candidatus Vesicomyosocius okutanii HA	171	172
Candidatus Ruthia magnifica str. Cm (Calyptogena	173	174
magnifica)	175	1/4
Methylococcus capsulatus str. Bath	175	176
uncultured marine bacterium EB80_02D08	177	178
uncultured marine gamma proteobacterium	179	180
EBAC31A08	1/9	180
uncultured marine gamma proteobacterium	181	182
EBAC20E09	101	162
uncultured gamma proteobacterium eBACHOT4E07	183	184
Alcanivorax borkumensis SK2	185	186
Chromohalobacter salexigens DSM 3043	187	188
Marinobacter algicola DG893	189	190
Marinobacter aquaeolei VT8	191	192
Marinobacter sp. ELB17	193	194
Pseudoalteromonas haloplanktis TAC125	195	196
Acinetobacter sp. ADP1	197	198
Opitutaceae bacterium TAV2	199	200
Flavobacterium sp. MED217	201	202
Cellulophaga sp. MED134	203	204
Kordia algicida OT-1	205	206
Flavobacteriales bacterium ALC-1	207	208
Psychroflexus torquis ATCC 700755	209	210
Flavobacteriales bacterium HTCC2170	211	212
unidentified eubacterium SCB49	213	214
Gramella forsetii KT0803	215	216
Robiginitalea biformata HTCC2501	217	218
Tenacibaculum sp. MED152	219	220
Polaribacter irgensii 23-P	221	222
Pedobacter sp. BAL39	223	224
Flavobacteria bacterium BAL38	225	226
Flavobacterium psychrophilum JIP02/86	227	228

Flavobacterium johnsoniae UW101	229	230
Lactococcus lactis subsp. cremoris SK11	231	232
Psychromonas ingrahamii 37	233	234
Microscilla marina ATCC 23134	235	236
Cytophaga hutchinsonii ATCC 33406	237	238
Rhodopirellula baltica SH 1	239	240
Blastopirellula marina DSM 3645	241	242
Planctomyces maris DSM 8797	243	244
Algoriphagus sp. PR1	245	246
Candidatus Sulcia muelleri str. Hc (Homalodisca coagulata)	247	248
Candidatus Carsonella ruddii PV	249	250
Synechococcus sp. RS9916	251	252
Synechococcus sp. WH 7803	253	254
Synechococcus sp. CC9311	255	256
Synechococcus sp. CC9605	257	258
Synechococcus sp. WH 8102	259	260
Synechococcus sp. BL107	261	262
Synechococcus sp. RCC307	263	264
Synechococcus sp. RS9917	265	266
Synechococcus sp. WH 5701	267	268
Prochlorococcus marinus str. MIT 9313	269	270
Prochlorococcus marinus str. NATL2A	271	272
Prochlorococcus marinus str. MIT 9215	273	274
Prochlorococcus marinus str. AS9601	275	276
Prochlorococcus marinus str. MIT 9515	277	278
Prochlorococcus marinus subsp. pastoris str. CCMP1986	279	280
Prochlorococcus marinus str. MIT 9211	281	282
Prochlorococcus marinus subsp. marinus str.	282	204
CCMP1375	283	284
Nodularia spumigena CCY9414	285	286
Nostoc punctiforme PCC 73102	287	288
Nostoc sp. PCC 7120	289	290
Trichodesmium erythraeum IMS101	291	292
Acaryochloris marina MBIC11017	293	294
Lyngbya sp. PCC 8106	295	296

Synechocystis sp. PCC 6803	297	298
Cyanothece sp. CCY0110	299	300
Thermosynechococcus elongatus BP-1	301	302
Synechococcus sp. JA-2-3B'a(2-13)	303	304
Gloeobacter violaceus PCC 7421	305	306
Nitrosomonas eutropha C91	307	308
Nitrosomonas europaea ATCC 19718	309	310
Nitrosospira multiformis ATCC 25196	311	312
Chloroflexus aggregans DSM 9485	313	314
Leptospirillum sp. Group II UBA	315	316
Leptospirillum sp. Group II UBA	317	318
Halorhodospira halophila SL1	319	320
Nitrococcus mobilis Nb-231	321	322
Alkalilimnicola ehrlichei MLHE-1	323	324
Deinococcus geothermalis DSM 11300	325	326
Polynucleobacter sp. QLW-P1DMWA-1	327	328
Polynucleobacter necessarius STIR1	329	330
Azoarcus sp. EbN1	331	332
Burkholderia phymatum STM815	333	334
Burkholderia xenovorans LB400	335	336
Burkholderia multivorans ATCC 17616	337	338
Burkholderia cenocepacia PC184	339	340
Burkholderia mallei GB8 horse 4	341	342
Ralstonia eutropha JMP134	343	344
Ralstonia metallidurans CH34	345	346
Ralstonia solanacearum UW551	347	348
Ralstonia pickettii 12J	349	350
Limnobacter sp. MED105	351	352
Herminiimonas arsenicoxydans	353	354
Bordetella parapertussis	355	356
Bordetella petrii DSM 12804	357	358
Polaromonas sp. JS666	359	360
Polaromonas naphthalenivorans CJ2	361	362
Rhodoferax ferrireducens T118	363	364
Verminephrobacter eiseniae EF01-2	365	366
Acidovorax sp. JS42	367	368
Delftia acidovorans SPH-1	369	370
Methylibium petroleiphilum PM1	371	372

gamma proteobacterium KT 71	373	374
Tremblaya princes	375	376
Blastopirellula marina DSM 3645	377	378
Planctomyces maris DSM 8797	379	380
Microcystis aeruginosa PCC 7806	381	382
Salinibacter ruber DSM 13855	383	384
Methylobacterium chloromethanicum	385	386

Table 4b. Additional representative bacterial [2Fe-2S] DHAD proteins and encoding sequences.

Organism of derivation	Nucleic acid	Amino acid
	SEQ ID NO:	SEQ ID NO:
Burkholderia ambifaria AMMD	387	388
Bradyrhizobium sp. BTAil	389	390
Delftia acidovorans SPH-1	391	392
Microcystis aeruginosa NIES-843	393	394
uncultured marine microorganism HF4000_APKG8C21	395	396
Burkholderia ubonensis Bu	397	398
Gemmata obscuriglobus UQM 2246	399	400
Mycobacterium abscessus	401	402
Synechococcus sp. PCC 7002	403	404
Burkholderia graminis C4D1M	405	406
Methylobacterium radiotolerans JCM 2831	407	408
Leptothrix cholodnii SP-6	409	410
Verrucomicrobium spinosum DSM 4136	411	412
Cyanothece sp. ATCC 51142	413	414
Opitutus terrae PB90-1	415	416
Leptospira biflexa serovar Patoc strain 'Patoc 1 (Paris)'	417	418
Methylacidiphilum infernorum V4	419	420
Cupriavidus taiwanensis	421	422
Chthoniobacter flavus Ellin428	423	424
Cyanothece sp. PCC 7822	425	426
Phenylobacterium zucineum HLK1	427	428
Leptospirillum sp. Group II '5-way CG'	429	430
Arthrospira maxima CS-328	431	432
Oligotropha carboxidovorans OM5	433	434
Rhodospirillum centenum SW	435	436
Cyanothece sp. PCC 8801	437	438

Thermus aquaticus Y51MC23	439	440
Cyanothece sp. PCC 7424	441	442
Acidithiobacillus ferrooxidans ATCC 23270	443	444
Cyanothece sp. PCC 7425	445	446
Arthrobacter chlorophenolicus A6	447	448
Burkholderia multivorans CGD2M	449	450
Thermomicrobium roseum DSM 5159	451	452
bacterium Ellin514	453	454
Desulfobacterium autotrophicum HRM2	455	456
Thioalkalivibrio sp. K90mix	457	458
Flavobacteria bacterium MS024-3C	459	460
Flavobacteria bacterium MS024-2A	461	462
'Nostoc azollae' 0708	463	464
Acidobacterium capsulatum ATCC 51196	465	466
Gemmatimonas aurantiaca T-27	467	468
Gemmatimonas aurantiaca T-27	469	470
Rhodococcus erythropolis PR4	471	472
Deinococcus deserti VCD115	473	474
Rhodococcus opacus B4	475	476
Chryseobacterium gleum ATCC 35910	477	478
Thermobaculum terrenum ATCC BAA-798	479	480
Kribbella flavida DSM 17836	481	482
Gordonia bronchialis DSM 43247	483	484
Geodermatophilus obscurus DSM 43160	485	486
Xylanimonas cellulosilytica DSM 15894	487	488
Sphingobacterium spiritivorum ATCC 33300	489	490
Meiothermus silvanus DSM 9946	491	492
Meiothermus ruber DSM 1279	493	494
Nakamurella multipartita DSM 44233	495	496
Cellulomonas flavigena DSM 20109	497	498
Rhodothermus marinus DSM 4252	499	500
Planctomyces limnophilus DSM 3776	501	502
Beutenbergia cavernae DSM 12333	503	504
Spirosoma linguale DSM 74	505	506
Sphaerobacter thermophilus DSM 20745	507	508
Lactococcus lactis	509	510
Thermus thermophilus HB8	511	512
Anabaena variabilis ATCC 29413	513	514

Roseovarius sp. 217	515	516
uncultured Prochlorococcus marinus clone HF10-88D1	517	518
Burkholderia xenovorans LB400	519	520
Saccharomonospora viridis DSM 43017	521	522
Pedobacter heparinus DSM 2366	523	524
Microcoleus chthonoplastes PCC 7420	525	526
Acidimicrobium ferrooxidans DSM 10331	527	528
Rhodobacterales bacterium HTCC2083	529	530
Candidatus Pelagibacter sp. HTCC7211	531	532
Chitinophaga pinensis DSM 2588	533	534
Alcanivorax sp. DG881	535	536
Micrococcus luteus NCTC 2665	537	538
Verrucomicrobiae bacterium DG1235	539	540
Synechococcus sp. PCC 7335	541	542
Brevundimonas sp. BAL3	543	544
Dyadobacter fermentans DSM 18053	545	546
gamma proteobacterium NOR5-3	547	548
gamma proteobacterium NOR51-B	549	550
Cyanobium sp. PCC 7001	551	552
Jonesia denitrificans DSM 20603	553	554
Brachybacterium faecium DSM 4810	555	556
Paenibacillus sp. JDR-2	557	558
Octadecabacter antarcticus 307	559	560
Variovorax paradoxus S110	561	562

Table 5. SEQ ID NOs of Representative Fungal and Plant [2Fe-2S] DHAD Proteins and Encoding Sequences.

Description	SEQ ID NO:	SEQ ID NO:
	Nucleic acid	Peptide
Schizosaccharomyces pombe ILV3	563	564
Saccharomyces cerevisiae ILV3	565	566
Kluyveromyces lactis ILV3	567	568
Candida albicans SC5314 ILV3	569	570
Pichia stipitis CBS 6054 ILV3	571	572
Yarrowia lipolytica ILV3	573	574
Candida galbrata CBS 138 ILV3	575	576
Chlamydomonas reinhardtii	577	578

Ostreococcus lucimarinus CCE9901	579	580
Vitis vinifera		
(Unnamed protein product: CAO71581.1)	581	582
Vitis vinifera		
(Hypothetical protein: CAN67446.1)	583	584
Arabidopsis thaliana	585	586
Oryza sativa (indica cultivar-group)	587	588
Physcomitrella patens subsp. Patens	589	590
Chaetomium globosum CBS 148.51	591	592
Neurospora crassa OR74A	593	594
Magnaporthe grisea 70-15	595	596
Gibberella zeae PH-1	597	598
Aspergillus niger	599	600
Neosartorya fischeri NRRL 181		
(XP_001266525.1)	601	602
Neosartorya fischeri NRRL 181		
(XP_001262996.1)	603	604
Aspergillus niger		
(hypothetical protein An03g04520)	605	606
Aspergillus niger		
(Hypothetical protein An14g03280)	607	608
Aspergillus terreus NIH2624	609	610
Aspergillus clavatus NRRL 1	611	612
Aspergillus nidulans FGSC A4	613	614
Aspergillus oryzae	615	616
Ajellomyces capsulatus NAm1	617	618
Coccidioides immitis RS	619	620
Botryotinia fuckeliana B05.10	621	622
Phaeosphaeria nodorum SN15	623	624
Pichia guilliermondii ATCC 6260	625	626
Debaryomyces hansenii CBS767	627	628
Lodderomyces elongisporus NRRL YB-4239	629	630
Vanderwaltozyma polyspora DSM 70294	631	632
Ashbya gossypii ATCC 10895	633	634
Laccaria bicolor S238N-H82	635	636
Coprinopsis cinerea okayama7#130	637	638
Cryptococcus neoformans var. neoformans JEC21	639	640
Ustilago maydis 521	641	642

Malassezia globosa CBS 7966	643	644
Aspergillus clavatus NRRL 1	645	646
Neosartorya fischeri NRRL 181		
(Putative)	647	648
Aspergillus oryzae	649	650
Aspergillus niger (hypothetical protein An18g04160)	651	652
Aspergillus terreus NIH2624	653	654
Coccidioides immitis RS (hypothetical protein		
CIMG_04591)	655	656
Paracoccidioides brasiliensis	657	658
Phaeosphaeria nodorum SN15	659	660
Gibberella zeae PH-1	661	662
Neurospora crassa OR74A	663	664
Coprinopsis cinerea okayama 7#130	665	666
Laccaria bicolor S238N-H82	667	668
Ustilago maydis 521	669	670

Table 6. SEQ ID NOs of Representative [4Fe-4S] DHAD Proteins and Encoding Sequences.

Organism	SEQ ID NO:	SEQ ID NO:
	Nucleic acid	Peptide
Escherichia coli str. K-12 substr. MG1655	671	672
Bacillus subtilis subsp. subtilis str. 168	673	674
Agrobacterium tumefaciens str. C58	675	676
Burkholderia cenocepacia MC0-3	677	678
Psychrobacter cryohalolentis K5	679	680
Psychromonas sp. CNPT3	681	682
Deinococcus radiodurans R1	683	684
Wolinella succinogenes DSM 1740	685	686
Zymomonas mobilis subsp. mobilis ZM4	687	688
Clostridium acetobutylicum ATCC 824	689	690
Clostridium beijerinckii NCIMB 8052	691	692
Pseudomonas fluorescens Pf-5	693	694
Methanococcus maripaludis C7	695	696
Methanococcus aeolicus Nankai-3	697	698
Vibrio fischeri ATCC 700601 (ES114)	699	700
Shewanella oneidensis MR-1 ATCC 700550	701	702

[0098] Additional [2Fe-2S] DHADs may be identified using the analysis described in U.S. Patent Appl. No. 12/569,636, filed Sept. 29, 2009, which is herein incorporated by reference. The analysis is as follows: A Profile Hidden Markov Model (HMM) was prepared based on amino acid sequences of eight functionally verified DHADs. The application of Profile HMM has been described. See, e.g., Krogh et al., J. Mol. Biol. 235:1501-1531 (1994) and Durbin et al., "Markov chains and hidden Markov models," in Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids, Cambridge University Press (1998). A Profile HMM is a statistical model built of multiple sequence alignments that can be used to determine whether or not a test sequence belongs to a particular family of sequences. See id. A Profile HMM can be built by first generating an alignment of functionally verified sequences using conventional sequence alignment tools. Next, the sequence alignment is used to build the Profile HMM using publicly available software programs (e.g., HMMER) that use a position-specific scoring system to capture information about the degree of conservation at various amino acid positions in the multiple alignment of the input sequences. More specifically, the scores of amino acid residues in a "match" state (i.e., match state emission scores), or in an "insert" state (i.e., insert state emission scores) are captured which are proportional to the expression: Log = 2 (p x)/(null x). See id. In this expression, the term "p x" is the probability of an amino acid residue, at a particular position in the alignment, according to the Profile HMM, and the term "null x" is the probability according to the Null model. See id. The Null model is a simple one state probabilistic model with a pre-calculated set of emission probabilities for each of the amino acids derived from the distribution of amino acids. See id. "State" transition scores are also calculated as log odds parameters and are proportional to Log = 2 (t x). See id. In this expression, the term "t x" is the probability of transiting to an emitter or non-emitter state. See id. Further details regarding the particular statistical analyses to generate a Profile HMM are available in Krogh et al., J. Mol. Biol. 235:1501-1531 (1994) and Durbin et al., "Markov chains and hidden Markov models," in Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids, Cambridge University Press (1998), and U.S. Patent Appl. No. 12/569,636.

[0099]

A Profile Hidden Markov Model (HMM) was prepared based on amino acid sequences of eight functionally verified DHADs are from *Nitrosomonas europaea* (DNA

SEQ ID NO:309; protein SEQ ID NO:310), Synechocystis sp. PCC6803 (DNA SEQ ID:297; protein SEQ ID NO:298), Streptococcus mutans (DNA SEQ ID NO:167; protein SEQ ID NO:168), Streptococcus thermophilus (DNA SEQ ID NO:163; SEQ ID No:164), Ralstonia metallidurans (DNA SEQ ID NO:345; protein SEQ ID NO:346), Ralstonia eutropha (DNA SEQ ID NO:343; protein SEQ ID NO:344), and Lactococcus lactis (DNA SEQ ID NO:231; protein SEQ ID NO:232). In addition the DHAD from Flavobacterium johnsoniae (DNA SEQ ID NO:229; protein SEQ ID NO:230) was found to have dihydroxy-acid dehydratase activity when expressed in E. coli and was used in making the Profile. The Profile HMM is prepared using the HMMER software package (The theory behind profile HMMs is described in R. Durbin, S. Eddy, A. Krogh, and G. Mitchison, Biological sequence analysis: probabilistic models of proteins and nucleic acids, Cambridge University Press, 1998; Krogh et al., 1994; J. Mol. Biol. 235:1501-1531), following the user guide which is available from HMMER (Janelia Farm Research Campus, Ashburn, VA). The output of the HMMER software program is a Profile Hidden Markov Model (HMM) that characterizes the input sequences. The Profile HMM prepared for the eight DHAD proteins is given in U.S. Appl. No. 12/569,636, filed Sept. 29, 2009 and in Table 12.

- [00100] The first line in Table 12 for each position reports the probability for each amino acid to be in that "state" (match state emission scores). The second line reports the insert state emission scores, and the third line reports the state transition scores. The highest probability is highlighted for each position. These scores can be converted into "E values" (expectation values), which are the number of hits or matches to the Profile HMM one would expect to obtain just by chance. A protein having an E value of $< 10^{-5}$ match to the Profile HMM, indicates that the protein shares significant sequence similarity with the seed proteins used to construct the Profile HMM and that the protein belongs to the family represented by the profile HMM.
- [0100] Any protein that matches the Profile HMM with an E value of $< 10^{-5}$ is a DHAD related protein, which includes [4Fe-4S] DHADs, [2Fe-2S] DHADs, arabonate dehydratases, and phosphogluconate dehydratases. In embodiments, sequences matching the Profile HMM are then analyzed for the presence of the three conserved cysteines, corresponding to positions 56, 129, and 201 in the *Streptococcus mutans* DHAD. The presence of all three conserved cysteines is characteristic of proteins having a [2Fe-2S]

cluster. Proteins having the three conserved cysteines include arabonate dehydratases and [2Fe-2S] DHADs. The [2Fe-2S] DHADs may be distinguished from the arabonate dehydratases by analyzing for signature conserved amino acids found to be present in the [2Fe-2S] DHADs or in the arabonate dehydratases at positions corresponding to the following positions in the *Streptococcus mutans* DHAD amino acid sequence. These signature amino acids are in [2Fe-2S] DHADs or in arabonate dehydratases, respectively, at the following positions (with greater than 90% occurance): 88 asparagine vs. glutamic acid; 113 not conserved vs. glutamic acid; 142 arginine or asparagine vs. not conserved; 165 not conserved vs. glycine; 208 asparagine vs. not conserved; 454 leucine vs. not conserved; 477 phenylalanine or tyrosine vs. not conserved; and 487 glycine vs. not conserved.

- [0101] Additionally, the sequences of DHAD coding regions provided herein may be used to identify other homologs in nature. Such methods are well-known in the art, and various methods that may be used to isolate genes encoding homologous proteins are described in U.S. Appl. No. 12/569,636, filed Sept. 29, 2009, which such methods are incorporated by reference herein.
- **[0102]** The presence of DHAD activity in a cell engineered to express a heterologous DHAD can be confirmed using methods known in the art. As one example, and as demonstrated in the Examples herein, crude extracts from cells engineered to express a bacterial DHAD may be used in a DHAD assay as described by Flint and Emptage (*J. Biol. Chem.* (1988) 263(8): 3558-64) using dinitrophenylhydrazine. In another example, DHAD activity may be assayed by expressing a heterologous DHAD identifiable by the methods disclosed herein in a yeast strain that lacks endogenous DHAD activity. If DHAD activity is present, the yeast strain will grow in the absence of branched-chain amino acids. DHAD activity may also be confirmed by more indirect methods, such as by assaying for a downstream product in a pathway requiring DHAD activity. Any product that has α -ketoisovalerate or α -ketomethylvalerate as a pathway intermediate may be measured in an assay for DHAD activity. A list of such products includes, but is not limited to, valine, isoleucine, leucine, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol.
- [0103] Over-Expression of DHAD Activity

- [0104] Applicants have found that expression of a heterologous DHAD can provide DHAD activity when expressed in a host cell. Expression of a DHAD which may be identified as described herein can provide DHAD activity for a biosynthetic pathway that includes conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate or 2,3-dihydroxymethylvalerate to α -ketomethylvalerate. In addition, the *S. mutans* [2Fe-2S] DHAD was shown in related U.S. Appl. No. 12/569,636, filed Sept. 29, 2009, incorporated by reference herein, to have higher stability in air as compared to the sensitivity in air of the E. coli [4Fe-4S] DHAD, which is desirable for obtaining better activity in a heterologous host cell.
- [0105] Furthermore, as described herein, it has been found that expressing a heterologous DHAD protein at higher levels can provide increased DHAD activity when expressed in a host cell. High expression of a recombinant polynucleotide can be accomplished in at least two ways: 1) by increasing the copy number of a plasmid comprising the recombinant polynucleotide; or 2) by integrating multiple copies of the gene of interest into the host cell's chromosome. As exemplified herein, expression of multiple copies of the heterologous DHAD, provides an increase in specific activity of heterologous DHAD
- [0106] Recombinant polynucleotides are typically cloned for expression using the coding sequence as part of a chimeric gene used for transformation, which includes a promoter operably linked to the coding sequence as well as a ribosome binding site and a termination control region. The coding region may be from the host cell for transformation and combined with regulatory sequences that are not native to the natural gene encoding DHAD. Alternatively, the coding region may be from another host cell.
- [0107] Vectors useful for the transformation of a variety of host cells are common and described in the literature. Typically the vector contains a selectable marker and sequences allowing autonomous replication or chromosomal integration in the desired host. In addition, suitable vectors may comprise a promoter region which harbors transcriptional initiation controls and a transcriptional termination control region, between which a coding region DNA fragment may be inserted, to provide expression of the inserted coding region. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the specific species chosen as a production host.

- [0108] Yeast cells that can be hosts for expression or over-expression of a heterologous bacterial DHAD are any yeast cells that are amenable to genetic manipulation and include, but are not limited to, *Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia*, and *Pichia*. Suitable strains include, but are not limited to, *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces thermotolerans, Candida glabrata, Candida albicans, Pichia stipitis* and *Yarrowia lipolytica*. In one embodiment, the host is *Saccharomyces cerevisiae*.
- [0109] Expression is achieved by transforming a host cell with a gene comprising a sequence encoding DHAD, for example, a DHAD listed in Tables 4a, 4b, 5 or 6, or identified using the screening methods in related U.S. Appl. No. 12/569,636, filed Sept. 29, 2009, incorporated by reference herein. The coding region for the DHAD to be expressed may be codon optimized for the target host cell, as well known to one skilled in the art. Methods for gene expression in yeast are known in the art (see, e.g., Methods in Enzymology, Volume 194, Guide to Yeast Genetics and Molecular and Cell Biology (Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, CA). Expression of genes in yeast typically requires a promoter, operably linked to a coding region of interest, and a transcriptional terminator. A number of yeast promoters can be used in constructing expression cassettes for genes in yeast, including, but not limited to, promoters derived from the following genes: CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI, CUP1, FBA, GPD, GPM, and AOX1. Suitable transcriptional terminators include, but are not limited to, FBAt, GPDt, GPMt, ERG10t, GAL1t, CYC1, and ADH1.
- **[0110]** Suitable promoters, transcriptional terminators, and DHAD coding regions may be cloned into *E. coli*-yeast shuttle vectors, and transformed into yeast cells. These vectors allow strain propagation in both *E. coli* and yeast strains. In one embodiment, the vector used contains a selectable marker and sequences allowing autonomous replication or chromosomal integration in the desired host. Examples of plasmids used in yeast are shuttle vectors pRS423, pRS424, pRS425, and pRS426 (American Type Culture Collection, Manassas, VA), which contain an *E. coli* replication origin (*e.g.*, pMB1), a yeast 2-micron origin of replication, and a marker for nutritional selection. The selection markers for these four vectors are His3 (vector pRS423), Trp1 (vector pRS424), Leu2

(vector pRS425) and Ura3 (vector pRS426). Construction of expression vectors with a chimeric gene encoding the described DHADs can be performed by either standard molecular cloning techniques in *E. coli* or by the gap repair recombination method in yeast.

- [0111] The gap repair cloning approach takes advantage of the highly efficient homologous recombination in yeast. For example, a yeast vector DNA is digested (e.g., in its multiple cloning site) to create a "gap" in its sequence. A number of insert DNAs of interest are generated that contain $a \ge 21$ bp sequence at both the 5' and the 3' ends that sequentially overlap with each other, and with the 5' and 3' terminus of the vector DNA. For example, to construct a yeast expression vector for "Gene X," a yeast promoter and a yeast terminator are selected for the expression cassette. The promoter and terminator are amplified from the yeast genomic DNA, and Gene X is either PCR amplified from its source organism or obtained from a cloning vector comprising Gene X sequence. There is at least a 21 bp overlapping sequence between the 5' end of the linearized vector and the promoter sequence, between the promoter and Gene X, between Gene X and the terminator sequence, and between the terminator and the 3' end of the linearized vector. The "gapped" vector and the insert DNAs are then co-transformed into a yeast strain and plated on the medium containing the appropriate compound mixtures that allow complementation of the nutritional selection markers on the plasmids. The presence of correct insert combinations can be confirmed by PCR mapping using plasmid DNA prepared from the selected cells. The plasmid DNA isolated from yeast (usually low in concentration) can then be transformed into an E. coli strain, e.g. TOP10, followed by mini preps and restriction mapping to further verify the plasmid construct. Finally, the construct can be verified by sequence analysis.
- **[0112]** Like the gap repair technique, integration into the yeast genome also takes advantage of the homologous recombination system in yeast. For example, a cassette containing a coding region plus control elements (promoter and terminator) and auxotrophic marker is PCR-amplified with a high-fidelity DNA polymerase using primers that hybridize to the cassette and contain 40-70 base pairs of sequence homology to the regions 5' and 3' of the genomic area where insertion is desired. The PCR product is then transformed into yeast and plated on medium containing the appropriate compound mixtures that allow selection for the integrated auxotrophic marker. For example, to

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integrate "Gene X" into chromosomal location "Y", the promoter-coding regionXterminator construct is PCR amplified from a plasmid DNA construct and joined to an autotrophic marker (such as URA3) by either SOE PCR or by common restriction digests and cloning. The full cassette, containing the promoter-coding regionX-terminator-URA3 region, is PCR amplified with primer sequences that contain 40-70 bp of homology to the regions 5' and 3' of location "Y" on the yeast chromosome. The PCR product is transformed into yeast and selected on growth media lacking uracil. Transformants can be verified either by colony PCR or by direct sequencing of chromosomal DNA.

[0113]

In addition to the above materials and methods that may be used to express a heterologous DHAD, these same, or similar, materials and methods may be used to overexpress a heterologous DHAD using modifications known to one of skill in the art. For example, when using a plasmid-based system to over-express the recombinant polynucleotide, a high-copy number vector, or a vector with a copy number that can be regulated, may be constructed. Such a regulatable or inducible system is described herein in Example 1; however, other systems are known to one of skill in the art and may be used to construct other high-copy number or copy number regulatable vectors. Alternatively, when using an integration-based system to over-express the recombinant polypeptide, an integration vector is required for targeting at multiple integration sites. A multiple integration-based system is described herein in Example 2; however, other multiple integration-based systems are known to one of skill in the art and may be used to target multiple integrations of a recombinant polypeptide, for example integration into rDNA regions.

[0114] Expression of the heterologous DHAD in the recombinant host cell can be quantified, e.g., by a percent of total cell protein. Such over-expression can be quantified in an amount selected from the group consisting of: (a) greater than about 0.001% of total cell protein; (b) greater than about 0.01% of total cell protein; (c) greater than about 0.1%of total cell protein; (d) greater than about 0.5% of total cell protein; (e) greater than about 1.0% of total cell protein; (f) greater than about 2.0% of total cell protein; (g) greater than about 5% of total cell protein; (h) greater than about 10% of total cell protein; and (i) greater than about 20% of total cell protein.

[0115] The specific activity of the heterologous DHAD produced in a recombinant host cell can be quantified, e.g., as U/mg. The heterologous DHAD specific activity can be - 47 -

selected from the group consisting of: (a) greater than about 0.25 U/mg; (b) greater than about 0.3 U/mg; (c) greater than about 0.5 U/mg; (d) greater than about 1.0 U/mg; (e) greater than about 1.5 U/mg; (f) greater than about 2.0 U/mg; (g) greater than about 3.0 U/mg; (h) greater than about 4.0 U/mg; (i) greater than about 5.0 U/mg; (j) greater than about 6.0 U/mg; (k) greater than about 7.0 U/mg; (l) greater than about 8.0 U/mg; (m) greater than about 9.0 U/mg; (n) greater than about 10.0 U/mg; (o) greater than about 20.0 U/mg; and (p) greater than about 50.0 U/mg.

- [0116] The heterologous DHAD specific activity can also be quantified, e.g., as a percent comparison to an endogenous DHAD specific activity or to some other control DHAD specific activity. An example of a "control" DHAD specific activity is that from a heterologous DHAD expressed in a recombinant host cell using a low copy number plasmid or a plasmid that is not other wise inducible or regulatable. Such a control establishes a baseline from which to compare the specific activity of the same heterologous DHAD expressed in a recombinant host cell using a high copy number plasmid or a plasmid with copy number that can be regulated, or co-expressed with polynucleotides encoding polypeptides affecting Fe-S cluster biosynthesis or Fe uptake and utilization, as described below. Thus, the increase in specific activity of the heterologous DHAD when compared to the control DHAD specific activity can be in an amount selected from the group consisting of: greater than an about 10% increase; greater than an about 20% increase; greater than an about 30% increase; greater than an about 40% increase; greater than an about 50% increase; greater than an about 60% increase; greater than an about 70% increase; greater than an about 80% increase; greater than an about 90% increase; greater than an about 95% increase; greater than an about 98% increase; and greater than an about 99% increase. The heterologous DHAD specific activity can also be expressed by "fold increase" over control. Thus, the increase in specific activity can be selected from the group consisting of: (a) greater than about 2-fold higher, (b) greater than about 5-fold higher, (c) greater than about 8-fold higher, or (d) greater than about 10-fold higher than control.
- [0117]

Fe-S Cluster Forming Proteins and Fe Regulation, Utilization, and Homeostasis

[0118] As described above, DHAD enzymes require Fe-S clusters for functioning, therefore, they must be expressed in a host having the genetic machinery to produce and load Fe-S clusters into the apo-protein if they are going to be expressed in functional

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As described elsewhere herein, in normal yeast, the mitochondria play an form. important role in Fe-S cluster biosynthesis. The flux in the formation and movement of Fe-S cluster precursors from mitochondria to Fe-S cluster requiring proteins in the cytosol of normal yeast is believed to be limited. For example, after a point a further increase in the expression of the protein of heterologous DHADs in the cytosol does not result in a corresponding increase in DHAD activity. While not wishing to be bound by theory, it is believed that this is because the increased amounts of the heterologous DHAD are not getting loaded with the Fe-S cluster requisite for activity because the cell is not able to supply the increased demand for Fe-S clusters that arises in the conditions described above. Demonstrated herein is that yeast cells can be genetically modified in 2 ways (separately or contemporaneously) that will result in an increased fraction of the heterologous DHAD expressed in the cytosol being loaded with its requisite Fe-S cluster. One way is to to modify the expression of yeast genes involved in the Fe-S cluster formation, such as Fe-S cluster biosynthesis pathway genes or Fe uptake and utilization genes. The other way is to express heterologous genes involved in Fe-S cluster biosynthesis or Fe uptake and utilization in the cytoplasm of yeast.

- [0119] Yeast genes that encode polypeptides that are involved in Fe uptake and utilization and Fe-S cluster biosynthesis are candidates for modification of expression. In embodiments, the modification results in increased function of a selected Fe-S cluster requiring protein.
- [0120] As an example, Aft1 has been found to act as a transcriptional activator for genes into the iron regulon (Kumanovics, et al. J. Biol. Chem., 2008. 283, p. 10276-10286; Li, H., et al., The Yeast Iron Regulatory Proteins Grx3/4 and Fra2 form Heterodimeric Complexes Containing a [2Fe-2S] Cluster with Cysteinyl and Histidyl Ligation. Biochemistry, 2009. 48(40): p. 9569-9581. As exemplified herein, the deletion of known inhibitors of Aft1 translocation, results in an increase in specific activity of an Fe-S cluster requiring protein because it leads to an increase Fe-S cluster loading of the protien. While not wishing to be bound by theory, it is thus believed that altering expression of certain genes of the Fe regulon, whether directly or through deletion or upregulation of inhibitors, will likewise increase the loading and function of Fe-S cluster requiring proteins. For example, genes that play a role in, or are part of, Fe utilization and homeostasis in yeast, such as Fe Regulon genes, may be targeted for altered

expression. Such genes are known in the art, and examples of these genes are listed in Table 7. (The list in Table 7 is taken from Rutherford, J.C., *et al.*, *Activation of the Iron Regulon by the Yeast Aft1/Aft2 Transcription Factors Depends on Mitochondrial but Not Cytosolic Iron-Sulfur Protein Biogenesis.*, *J. Biol. Chem.*, 2005. **280**(11): p. 10135-10140; Foury, F. and D. Talibi, *Mitochondrial control of iron homeostasis. A genome wide analysis of gene expression in a yeast frataxin-deficient strain. J. Biol. Chem.*, 2001. **276**(11): p. 7762-7768; and Shakoury-Elizeh, M., *et al.*, *Transcriptional remodeling in response to iron deprivation in Saccharomyces cerevisiae. Mol. Biol. Cell*, 2004. **15**(3): p. 1233-1243.)

Table 7. Examples of yeast genes associated with Fe uptake and utilization .

Gene Name	Putative Function		Amino Acid	
		SEQ ID NO:	SEQ ID NO:	
ARNI	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to ferrirubin, ferrirhodin, and related siderophores	805	738	
ARN2	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to the siderophore triacetylfusarinine C	806	739	
ATX1	Cytosolic copper metallochaperone that transports copper to the secretory vesicle copper transporter Ccc2p for eventual insertion into Fet3p, which is a multicopper oxidase required for high-affinity iron uptake	802	735	
CCC2	Cu(+2)-transporting P-type ATPase, required for export of copper from the cytosol into an extracytosolic compartment; has similarity to human proteins involved in Menkes and Wilsons diseases	803	736	
COTI	Vacuolar transporter that mediates zinc transport into the vacuole; overexpression confers resistance to cobalt and rhodium	816	749	
ENB1 (ARN4)	Endosomal ferric enterobactin transporter, expressed under conditions of iron deprivation; member of the major facilitator superfamily; expression is regulated by Rcs1p and affected by chloroquine treatment	808	741	
FET3	Ferro-O2-oxidoreductase required for high-affinity iron uptake and involved in mediating resistance to copper ion toxicity, belongs to class of integral membrane multicopper oxidases	800	733	
FET5	Multicopper oxidase, integral membrane protein with similarity to Fet3p; may have a role in iron transport	814	747	
FIT1	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	792	725	
FIT2	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the	793	726	

	retention of siderophore-iron in the cell wall		
FIT3	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	794	727
FRE1	Ferric reductase and cupric reductase, reduces siderophore- bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels	795	728
FRE2	Ferric reductase and cupric reductase, reduces siderophore- bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels	796	729
FRE3	Ferric reductase, reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels	797	730
FRE4	Ferric reductase, reduces a specific subset of siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels	798	731
FRE5	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies	799	732
FRE6	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels	817	750
FTH1	Putative high affinity iron transporter involved in transport of intravacuolar stores of iron; forms complex with Fet5p; expression is regulated by iron; proposed to play indirect role in endocytosis	813	746
FTR1	High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with Fet3p; expression is regulated by iron	801	734
HMX1	ER localized, heme-binding peroxidase involved in the degradation of heme; does not exhibit heme oxygenase activity despite similarity to heme oxygenases; expression regulated by AFT1	823	756
SIT1 (ARN3)	Ferrioxamine B transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; transcription is induced during iron deprivation and diauxic shift; potentially phosphorylated by Cdc28p	807	740
SMF3	Putative divalent metal ion transporter involved in iron homeostasis; transcriptionally regulated by metal ions; member of the Nramp family of metal transport proteins	815	741
TIS11 (CTH2)	mRNA-binding protein expressed during iron starvation; binds to a sequence element in the 3'-untranslated regions of specific mRNAs to mediate their degradation; involved in iron homeostasis	824	757
VHTI	High-affinity plasma membrane H+-biotin (vitamin H) symporter; mutation results in fatty acid auxotrophy; 12 transmembrane domain containing major facilitator subfamily member; mRNA levels negatively regulated by iron deprivation and biotin	822	755

[0121] Based on their functions and association with Fe uptake and utilization, the proteins encoded by the genes disclosed in Table 7 are candidates for affecting Fe-S cluster biosynthesis. Additional yeast genes associated with Fe uptake and utilization or Fe-S cluster biosynthesis include those listed in Table 8.

Gene Name	Nucleic Acid SEQ ID NO:	Amino Acid SEQ ID NO:	Putative Function
AFT1	770	703	Transcription factor involved in iron utilization and homeostasis; binds the consensus site PyPuCACCCPu and activates the expression of target genes in response to changes in iron availability
AFT2	771	704	Iron-regulated transcriptional activator; activates genes involved in intracellular iron use and required for iron homeostasis and resistance to oxidative stress; similar to Aft1p
AIMI	779	712	Interacts with Grx3/4
ARHI	855	837	Oxidoreductase of the mitochondrial inner membrane, involved in cytoplasmic and mitochondrial iron homeostasis and required for activity of Fe-S cluster-containing enzymes; one of the few mitochondrial proteins essential for viability (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
ATM1	830	763	Mitochondrial inner membrane ATP-binding cassette (ABC) transporter, exports mitochondrially synthesized precursors of iron-sulfur (Fe/S) clusters to the cytosol (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
BUD32	778	711	Interacts with Grx3/4 and Aft1p
CADI	791	724	Stress responses including Fe deprivation; also regulates CTI6 and MRS4
(YAP2)			genes
CCCÍ	811	744	Putative vacuolar Fe2+/Mn2+ transporter; suppresses respiratory deficit of yfh1 mutants, which lack the ortholog of mammalian frataxin, by preventing mitochondrial iron accumulation
CFD1	834	767	Highly conserved, iron-sulfur cluster binding protein localized in the cytoplasm; forms a complex with Nbp35p that is involved in iron-sulfur protein assembly in the cytosol (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
CIA1	836	769	WD40 repeat protein involved in assembly of cytosolic and nuclear iron- sulfur proteins; similar to the human Ciaol protein; YDR267C is an essential gene (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
CMK1	784	717	Interacts with Grx4p
CTH1	825	758	mRNA binding and degradation under Fe depletion conditions
CTI6	786	719	Growth in low iron conditions
CYC8 (SSN6)	787	720	General transcriptional co-repressor, acts together with Tup1p; also acts as part of a transcriptional co-activator complex that recruits the SWI/SNF and SAGA complexes to promoters; can form the prion [OCT+]
DAP1	820	753	
DRE2	781	714	Interacts with Grx3p
ERV1	856	838	Flavin-linked sulfhydryl oxidase of the mitochondrial intermembrane space (IMS), oxidizes Mia40p as part of a disulfide relay system that promotes IMS retention of imported proteins; ortholog of human hepatopoietin (ALR) (<i>see</i> , <i>e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))

Table 8. Genes Associated With Yeast Fe Uptake and Utilization or Fe-S Cluster Biosynthesis

ESA1 FET4

FRA1

FRA2

GEF1

GGC1 (YHM1)

GRXI

		- 52 -
		Central players of the export pathway are the ABC transporter Atm1p of the mitochondrial inner membrane, the sulfhydryl oxidase Erv1p of the intermembrane space, and the tripeptide glutathione (23, 27, 50) (<i>see</i> Gerber, J., <i>et al.</i> , <i>Mol. Cell. Biol.</i> 24(11):4848-57 (2004))
782	715	Interacts with Grx4p/Aft1p
809	742	Low-affinity Fe(II) transporter of the plasma membrane
772	705	Protein involved in negative regulation of transcription of iron regulon; forms an iron independent complex with Fra2p, Grx3p, and Grx4p; cytosolic; mutant fails to repress transcription of iron regulon and is defective in spore formation
773	706	Protein involved in negative regulation of transcription of iron regulon; forms an iron independent complex with Fra2p, Grx3p, and Grx4p; null mutant fails to repress iron regulon and is sensitive to nickel
804	737	Copper transporter/loading for Fet3p
857	839	Mitochondrial GTP/GDP transporter, essential for mitochondrial genome maintenance; has a role in mitochondrial iron transport; member of the mitochondrial carrier family
858	840	Hydroperoxide and superoxide-radical responsive heat-stable glutathione- dependent disulfide oxidoreductase with active site cysteine pair; protects cells from oxidative damage
832	765	Cytoplasmic glutaredoxin, thioltransferase, glutathione-dependent disulfide

			cells from oxidative damage
GRX2	832	765	Cytoplasmic glutaredoxin, thioltransferase, glutathione-dependent disulfide oxidoreductase involved in maintaining redox state of target proteins, also exhibits glutathione peroxidase activity, expression induced in response to stress
GRX3	774	707	Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase; monothiol glutaredoxin subfamily member along with Grx4p and Grx5p; protects cells from oxidative damage
GRX4	775	708	Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase; monothiol glutaredoxin subfamily member along with Grx3p and Grx5p; protects cells from oxidative damage.
GRX5	831	764	Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase; mitochondrial matrix protein involved in the synthesis/assembly of iron-sulfur centers; monothiol glutaredoxin subfamily member along with Grx3p and Grx4p (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
HDA1	790	723	Interacts with Tup1p, Ssn6p for Aft1/2p regulation in the absence of heme
IBA57	859	841	Mitochondrial matrix protein involved in the incorporation of iron-sulfur clusters into mitochondrial aconitase-type proteins; activates the radical-SAM family members Bio2p and Lip5p; interacts with Ccr4p in the two-hybrid system (<i>see</i> , <i>e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
ISA1	860	842	Mitochondrial matrix protein involved in biogenesis of the iron-sulfur (Fe/S) cluster of Fe/S proteins, isal deletion causes loss of mitochondrial DNA and respiratory deficiency; depletion reduces growth on nonfermentable carbon sources (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))

ISA2	861	843	Protein required for maturation of mitochondrial and cytosolic Fe/S proteins, localizes to the mitochondrial intermembrane space, overexpression of ISA2 suppresses grx5 mutations (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
ISU1	828	761	Conserved protein of the mitochondrial matrix, performs a scaffolding function during assembly of iron-sulfur clusters, interacts physically and functionally with yeast frataxin (Yfh1p); isu1 isu2 double mutant is inviable (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
ISU2	829	762	Conserved protein of the mitochondrial matrix, required for synthesis of mitochondrial and cytosolic iron-sulfur proteins, performs a scaffolding function in mitochondria during Fe/S cluster assembly; isu1 isu2 double mutant is inviable (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
JACI	862	844	Specialized J-protein that functions with Hsp70 in Fe-S cluster biogenesis in mitochondria, involved in iron utilization; contains a J domain typical to J-type chaperones; localizes to the mitochondrial matrix (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
MGE1	863	845	Mitochondrial matrix cochaperone, acts as a nucleotide release factor for Ssc1p in protein translocation and folding; also acts as cochaperone for Ssq1p in folding of Fe-S cluster proteins; homolog of E. coli GrpE (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
MRS3	819	752	Iron transporter that mediates Fe2+ transport across the inner mitochondrial membrane; mitochondrial carrier family member, similar to and functionally redundant with Mrs4p; active under low-iron conditions; may transport other cations (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
MRS4	818	751	Iron transporter that mediates Fe2+ transport across the inner mitochondrial membrane; mitochondrial carrier family member, similar to and functionally redundant with Mrs3p; active under low-iron conditions; may transport other cations (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
MSN5	776	709	Exporting Aft1p and other proteins from the nucleus
NAR1	833	766	Component of the cytosolic iron-sulfur (FeS) protein assembly machinery, required for maturation of cytosolic and nuclear FeS proteins and for normal resistance to oxidative stress; homologous to human Narf (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
NBP35	835	768	Essential iron-sulfur cluster binding protein localized in the cytoplasm; forms a complex with Cfd1p that is involved in iron-sulfur protein assembly in the cytosol; similar to P-loop NTPases (<i>see</i> , <i>e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
NFS1	864	846	Cysteine desulfurase involved in iron-sulfur cluster (Fe/S) biogenesis; required for the post-transcriptional thio-modification of mitochondrial and cytoplasmic tRNAs; essential protein located predominantly in mitochondria (<i>see</i> , <i>e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
NFU1	865	847	Protein involved in iron utilization in mitochondria; similar to NifU, which is a protein required for the maturation of the Fe/S clusters of nitrogenase in

			nitrogen-fixing bacteria (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
NHP6a and b	788,789	721, 722	Both are high-mobility group non-histone chromatin protein, functionally redundant with Nhp6Bp; homologous to mammalian high mobility group proteins 1 and 2; acts to recruit transcription factor Rcs1p to certain promoters
PSE1	777	710	Importing Aft1p and other proteins to the nucleus
SMF1	810	743	Low affinity Fe(II) transporter of the plasma membrane
SNF1	866	848	AMP-activated serine/threonine protein kinase found in a complex containing Snf4p and members of the Sip1p/Sip2p/Gal83p family; required for transcription of glucose-repressed genes, thermotolerance, sporulation, and peroxisome biogenesis
SNF2	867	849	Catalytic subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; contains DNA-stimulated ATPase activity; functions interdependently in transcriptional activation with Snf5p and Snf6p
SNF3	868	850	Plasma membrane glucose sensor that regulates glucose transport; has 12 predicted transmembrane segments; long cytoplasmic C-terminal tail is required for low glucose induction of hexose transporter genes HXT2 and HXT4
SNF4	869	851	Activating gamma subunit of the AMP-activated Snf1p kinase complex (contains Snf1p and a Sip1p/Sip2p/Gal83p family member); activates glucose-repressed genes, represses glucose-induced genes; role in sporulation, and peroxisome biogenesis
SSQ1	827	760	Mitochondrial hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p, which is a homolog of human frataxin implicated in Friedreich's ataxia (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
TIM12 (MRS5)	871	853	Essential protein of the inner mitochondrial membrane, peripherally localized; component of the TIM22 complex, which is a twin-pore translocase that mediates insertion of numerous multispanning inner membrane protein.
TUP1	785	718	General repressor of transcription
NP_0119 11.1	821	754	
VPS41 (FET2)	872	854	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
YAHI	870	852	Ferredoxin of the mitochondrial matrix required for formation of cellular iron-sulfur proteins; involved in heme A biosynthesis; homologous to human adrenodoxin (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))
YAP5	812	745	Regulation (CCC1)
YFH1	826	759	Mitochondrial matrix iron chaperone; oxidizes and stores iron; interacts with
(Frataxin)			Isu1p to promote Fe-S cluster assembly; mutation results in multiple Fe/S- dependent enzyme deficiencies; human frataxin homolog is mutated in Friedrich's ataxia (<i>see, e.g.</i> , Lill, R. and U. Muehlenhoff, Ann. Rev. Biochem. 77:669-700 (2008))

YRA1	783	716	Interacts with Grx4p
ZPR1	780	713	Interacts with Aft1p

[0122] Additional genes encoding polypeptides affecting Fe-S cluster biosynthesis from other host cells have been identified and include, but are not limited to, those genes listed in Table 9.

Table 9. Genes Directly Involved in Fe-S Cluster Biosynthesis from Various Cells

Gene Name	Function
SEQ ID	(Accession; CDS)
NOs(Amino	
Acid, Nucleic	
Acid)	
	Azotobacter vinelandii nif genes
(Figures 6A a	and 6B; see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81 (2005))
iscA ^{nif}	[Fe-S] cluster scaffold protein (see Johnson, D.C., et al., Ann. Rev.
(873, 894)	Biochem. 74:247-81 (2005))
	(YP_002797399.1; nucleotides 153037 to 153360 of NC_012560.1)
nifU	NifU is a scaffold protein for assembly and transfer of iron-sulfur
(875, 896)	clusters (see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81
	(2005)).
	(YP_002797400.1; nucleotides 153425 to 154363 of NC_012560.1)
nifS	Cysteine desulfurase involved in the mobilization of S for nitrogenase
(874, 895)	maturation (see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81
	(2005)).
	(YP_002797401.1; nucleotides 154365 to 155573 of NC_012560.1)
cysE1	Involved in cysteine biosynthesis (see Johnson, D.C., et al., Ann. Rev.
(876, 897)	Biochem. 74:247-81 (2005))
	(YP_002797403.1; nucleotides 156797 to 157594 of NC_012560.1)
cysE2	Involved in cysteine biosynthesis (see Johnson, D.C., et al., Ann. Rev.
(929, 947)	Biochem. 74:247-81 (2005))
	(YP_002801153.1; reverse complement of nucleotides 4092159 to
	4092938 of NC_012560.1)
	Cysteine desulfurase involved in the mobilization of S (see Johnson,
	D.C., et al., Ann. Rev. Biochem. 74:247-81 (2005))
iscS	(YP_002801151.1; reverse complement of nucleotides of 4090290 to
(930, 948)	4091504 of NC_012560.1)
	[Fe-S] cluster scaffold protein (see Johnson, D.C., et al., Ann. Rev.
in a T T	Biochem. 74:247-81 (2005))
iscU	(YP_002801150.1; reverse complement of nucleotides 4089860 to
<u>(931, 949)</u>	4090246 of NC_012560.1)
iscA	[Fe-S] cluster scaffold protein (see Johnson, D.C., et al., Ann. Rev.
(932, 950)	Biochem. 74:247-81 (2005))

	(YP_002801149.1; reverse complement of nucleotides 4089511 to
	4089834 of NC_012560.1) HscB heat shock cognate protein associated with Isc-directed [Fe-S] protein maturation (see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81 (2005)) With the term of term o
hscB (933, 951)	(YP_002801148.1; reverse complement of nucleotides 4088980 to 4089501 of NC 012560.1)
	HscA heat shock cognate protein associated with Isc-directed [Fe-S] protein maturation (<i>see</i> Johnson, D.C., <i>et al.</i> , <i>Ann. Rev. Biochem.</i> 74:247-81 (2005))
hscA (934, 952)	(YP_002801147.1; reverse complement of nucleotides 4087072 to 4088937 of NC 012560.1)
Fdx (935, 953)	Ferredoxin (YP_002801146.1; reverse complement of nucleotides 4086730 to 4087071 of NC_012560.1)
sufS (936, 954)	Cysteine desulfurase involved in the mobilization of S (<i>see</i> Johnson, D.C., <i>et al.</i> , <i>Ann. Rev. Biochem.</i> 74:247-81 (2005)) (YP_002801025.1; nucleotides 3961166 to 3962515 of NC_012560.1)
sufE (937, 955)	(YP_002801026.1; nucleotides 3962512 to 3962916 of NC_012560.1)
cysE3 (938, 956)	Involved in cysteine biosynthesis (<i>see</i> Johnson, D.C., <i>et al.</i> , <i>Ann. Rev.</i> <i>Biochem.</i> 74:247-81 (2005)) (YP_002799274.1; nucleotides 2093069 to 2094052 of NC_012560.1)
sufS2 (939, 957)	Cysteine desulfurase involved in the mobilization of S (<i>see</i> Johnson, D.C., <i>et al.</i> , <i>Ann. Rev. Biochem.</i> 74:247-81 (2005)) (YP_002799276.1; nucleotides 2095267 to 2097081 of NC_012560.1)
<i>iscA2</i> also known as <i>eprA</i> (877, 898)	[Fe-S] cluster scaffold protein (<i>see</i> Johnson, D.C., <i>et al.</i> , <i>Ann. Rev.</i> <i>Biochem.</i> 74:247-81 (2005)) (YP_002801687.1; reverse complement of nucleotides 4681573 to 4681923 of NC 012560.1)
<i>Nfu</i> also known as <i>NfuA</i> (878, 899)	Human nfu appears to be a persulfide reductase according to the equation shown in Figure 6C. (<i>see</i> Liu, Y., W. Qi, and J.A. Cowan, <i>Biochem.</i> 48(5):973-80 (2009)) (YP_002800022.1; reverse complement of nucleotides 2961161 to 2961745 of NC_012560.1)
nfuA also known as AnfU (879, 900)	Spectroscopic and analytical studies indicate that one [4Fe-4S] cluster can be assembled <i>in vitro</i> within a dimeric form of NfuA. The resultant [4Fe-4S] cluster-loaded form of NfuA is competent for rapid in vitro activation of apo-aconitase. Based on these results a model is proposed where NfuA could represent a class of intermediate [Fe-S] cluster carriers involved in [Fe-S] protein maturation. (<i>see</i> Bandyopadhyay, S., <i>et al.</i> , <i>J Biol. Chem.</i> <i>283</i> (20):14092-99 (2008)) (YP_002801977.1; nucleotides 4963727 to 4964017 of NC_012560.1)
<i>nfuV</i> also known as <i>VnfU</i>	Could have specialized functions related to the maturation, protection, or repair of specific [Fe-S] proteins (<i>see</i> Johnson, D.C., <i>et al.</i> , <i>Ann. Rev.</i> <i>Biochem.</i> 74:247-81 (2005)).

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(880, 901)	(YP_002797514.1; reverse complement of nucleotides 263828 to 264118 of NC 012560.1)			
	Helicobacter pylori nif genes			
(Figure	7; see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81 (2005))			
nifS (881, 902)	NifS is a cysteine desulfurase.(YP_003057033.1; nucleotides 218891 to 220054 of NC_012973.1)			
nifU (882, 903)	NifU is a scaffold protein for assembly and transfer of iron-sulfur clusters. (YP_003057034.1; nucleotides 220076 to 221056 of NC_012973.1)			
nfu (927, 945)	$(P_003058109.1; nucleotides 1448886 to 1449155 of NC_012973.1)$			
iscS (928, 946)	(YP_003057709.1; reverse complement of nucleotides 1012615 to 1013937 of NC_012973.1)			
	E. coli isc genes			
(Figure	8; see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81 (2005))			
iscS (883, 904)	EcoCyc: IscS is a cysteine desulfurase that catalyzes the conversion of cysteine into alanine and sulfur via intermediate formation of a cysteine persulfide. (YP 026169.1; reverse complement of nucleotides 2658339 to 2659553			
	of NC_000913.2)			
iscU (884, 905)	EcoCyc: IscU is a scaffold protein for assembly and transfer of iron- sulfur clusters. IscU is able to form 2Fe-2S clusters and transfer them to apo-ferredoxin, acting catalytically. The chaperones HscA and HscB and ATP hydrolysis by HscA accelerate cluster transfer. (NP_417024.1; reverse complement of nucleotides 2657925 to 2658311 of NC_000913.2)			
iscA (885, 906)	EcoCyc: IscA is an iron-sulfur cluster assembly protein that forms the [2Fe-2S] cluster of ferredoxin. It has been shown to bind iron with an apparent association constant of $3 \times 10^{-19} \text{ M}^{-1}$. <i>In vitro</i> in the presence of IscS and cysteine, IscA can provide iron to iscU.			
	Native [2Fe-2S] SufA can transfer its Fe-S cluster to both [2Fe-2S] and [4Fe-4S] apoproteins. (<i>see</i> Gupta, V., <i>et al.</i> , <i>J. Am. Chem. Soc.</i> 131(17):6149-53 (2009))			
	The results suggest that the biogenesis of the [4Fe-4S] clusters and the [2Fe-2S] clusters may have distinct pathways and that IscA/SufA paralogues are essential for the [4Fe-4S] cluster assembly, but are dispensable for the [2Fe-2S] cluster assembly in E. coli under aerobic conditions. (Tan, G., <i>et al.</i> , <i>Biochem. J.</i> , 420(3):463-72 (2009)) (NP_417023.1; reverse complement of nucleotides 2657585 to 2657908 of NC_000913.2)			
hscB (886, 907)	EcoCyc: HscB is a co-chaperone that stimulates HscA (Hsc66) ATPase activity. HscB does not exhibit its own chaperone activity. HscB is required for wild-type stimulation of HscA ATPase activity by the			

	substants IsoII and for wild true interestion between IIsoA and IsoII
	substrate, IscU, and for wild-type interaction between HscA and IscU.
	This system is involved in iron-sulfur cluster assembly.
	(NP_417022.1; reverse complement of nucleotides 2656974 to 2657489 of NC_000913.2)
hscA	EcoCyc: Hsc66 together with Hsc20 may comprise a chaperone system
(887, 908)	similar to DnaK/DnaJ. Hsc66 is required for the assembly of iron-sulfur
	clusters. IscU may be a substrate for Hsc66. In the presence of Hsc20,
	IscU stimulates the ATPase activity of Hsc66 up to 480-fold; the <i>in vivo</i>
	turnover rate of the chaperone cycle may be determined by the
	availability of the IscU-Hsc20 complex. Hsc66 directly interacts with
	IscU, IscA, and Fdx.
	(NP_417021.1; reverse complement of nucleotides 2655107 to 2656957
	of NC_000913.2)
Fdx	EcoCyc: [2Fe-2S] ferridoxin
(888, 909)	(NP_417020.1; reverse complement of nucleotides 2654770 to 2655105
	of NC_000913.2)
	E. coli suf genes
(Figure	9; see Johnson, D.C., et al., Ann. Rev. Biochem. 74:247-81 (2005))
sufA	EcoCyc: SufA is part of the protein machinery that is involved in the
(889, 910)	biosynthesis of iron-sulfur clusters. In vitro, purified apoSufA can
	chelate iron-sulfur clusters by treatment with iron and sulfide under
	anaerobic conditions. HoloSufA then can form a fast and tight
	association with the target apoprotein biotin synthase (BioB) and
	transfers a [4Fe-4S] cluster to BioB in a slow reaction.
	(NP_416199.1; reverse complement of nucleotides 1762042 to 1762410
	of NC_000913.2)
sufB	EcoCyc: The SufB-SufC-SufD complex activates the cysteine
(890, 911)	desulfurase activity SufS in conjunction with the SufE sulfur acceptor
	protein.
	(NP_416198.2; reverse complement of nucleotides 1760546 to 1762033
	of NC_000913.2)
sufC	EcoCyc: SufC is part of the protein machinery that is involved in the
(891, 912)	biosynthesis of iron-sulfur clusters. The SufB-SufC-SufD complex
	activates the cysteine desulfurase activity of SufS in conjunction with
	the SufE sulfur acceptor protein.
	(NP_416197.1; reverse complement of nucleotides 1759790 to 1760536
	of NC_000913.2)
sufD	EcoCyc: The SufB-SufC-SufD complex activates the cysteine
(892, 913)	desulfurase activity SufS in conjunction with the SufE sulfur acceptor
	protein
	(NP_416196.1; reverse complement of nucleotides 1758544 to 1759815
	of NC_000913.2)
sufS	EcoCyc: SufS is a member of the NifS protein family. SufS exhibits
(893, 914)	activity with respect to assembly of the ferredoxin iron-sulfur cluster in
	an <i>in vitro</i> assay.
	(NP_416195.1; reverse complement of nucleotides 1757327 to 1758547
	of NC_000913.2)

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sufE1 also	(NP_416194.1; reverse complement of nucleotides 1756898 to 1757314
known as suf E	of NC_000913.2)
(925, 943)	
sufS2 also	(NP_417290.1; NC_000913.2 nucleotides 2941359 to 2942564)
known as csdA	
(924, 942)	
sufE2 also	(NP_417291.1; nucleotides 2942564 to 2943007 of NC_000913.2)
known as csdE	
(926, 944)	
iscA2 also	(NP_414698.1; nucleotides 176610 to 176954 of NC_000913.2)
known as erpA	
(922, 940)	
nfu also known	(NP_417873.1; nucleotides 3543646 to 3544221 of NC_000913.2)
as nfuA	
(923, 941)	

- **[0123]** Fe uptake and metabolism and/or Fe-S cluster biosynthesis genes, including, but not limited to, those listed in Tables 7, 8 or 9 can potentially be deleted, mutated, expressed, up-regulated, or down-regulated to increase the flux in an Fe-S cluster biosynthesis pathway and improve specific activity of Fe-S cluster requiring proteins such as DHAD. In addition, co-factors can be added to change the activity of polypeptides having Fe-S cluster regulatory activity to increase the flux in an Fe-S cluster biosynthesis pathway and improve DHAD specific activity.
- **[0124]** For example, the genes that increase the flux in an Fe-S cluster biosynthesis pathway can be expressed to improve the activity of DHAD by providing an adequate amount of Fe-S clusters for the apo-enzyme. Any gene, or a combination of them, such as one or more genes listed in Tables 7, 8, or 9, can be cloned and expressed in a pRS411 plasmid as described in Example 4. The resulting constructs, along with the DHAD expression vector pHR81 FBA ilvD(Sm), can then be transformed into wild-type BY4741. As a control, pRS411 without any gene of interest and vector pHR81 FBA ilvD(Sm) are transformed into a wild-type strain. The transformants are selected on agar plates with SD medium without uracil and methionine to maintain both plasmids as described in Example 4. Enzymatic activity for DHAD in the crude extract of different strains from the transformation can be measured. The results can be compared with the specific activity obtained from the control pRS411 without any gene of interest and vector pHR81 FBA ilvD(Sm) transformed into a wild-type strain. An increase in specific

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activity indicates a gene that can be used to increase the flux in an Fe-S cluster biosynthesis pathway.

- [0125] In addition, strains with deletions in more than one of the genes involved in Fe-S cluster regulatory activity can be created to provide additive effects in improving the enzymes or proteins containing Fe-S cluster(s). For example, double mutants with deletions in both *FRA2* and *GXR3* genes can be used to transform vector pHR81 FBA-IlvD(sm), and the DHAD activity in the crude extract from the transformants can be measured.
- [0126] Another alternative is to alter the expression of, e.g., the PSE1 (SEQ ID NO:777) gene, which encodes a protein involved in the import of Aft1p into the nucleus (Fukunaka, et al, 2003, J. Biological Chem., vol. 278, pp. 50120-50127). Expression of this gene can be accomplished by cloning it in vector pRS411 as described above.
- **[0127]** Thus, provided herein are recombinant host cells that comprise an alteration in the expression of any polypeptide encoded by an Fe uptake and utilization or an Fe-S cluster biosynthesis gene. Encompassed are recombinant host cells that comprise at least one heterologous polynucleotide of any one of the above-referenced Fe-S cluster biosynthesis genes. Also encompassed are recombinant host cells, wherein the host cell comprises at least one deletion, mutation, and/or substitution in an endogenous gene of any one of the above-referenced Fe uptake and utilization or Fe-S cluster biosynthesis genes. Also provided are recombinant host cells that comprise at least one heterologous polynucleotide of any one of the above-referenced Fe uptake and utilization or Fe-S cluster biosynthesis genes. Also provided are recombinant host cells that comprise at least one heterologous polynucleotide of any one of the above-referenced Fe uptake and utilization or Fe-S cluster biosynthesis genes, wherein the host cell comprises at least one heterologous polynucleotide of any one of the above-referenced Fe uptake and utilization or Fe-S cluster biosynthesis genes, wherein the host cell comprises at least one deletion, mutation, and/or substitution in an endogenous gene of any one of the above-referenced Fe uptake and utilization or Fe-S cluster biosynthesis genes.
- **[0128]** These recombinant host cells can also comprise at least one heterologous Fe-S cluster requiring protein. For example, provided herein is a recombinant host cell comprising at least one heterologous DHAD and at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis. Also provided is a recombinant host cell comprising at least one heterologous DHAD, wherein the host cell comprises at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis. Also provided is a recombinant host cell comprising at least one heterologous DHAD, wherein the host cell comprises at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis. Also provided is a recombinant host cell comprising at least one heterologous DHAD and at least one

heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis, wherein the host cell comprises at least one deletion, mutation, and/or substitution in an

- [0129] Host cells that can be used in the present invention include yeast host cells including, but not limited to, *Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia,* and *Pichia.* Bacterial host cells can also be used to create recombinant host cells that comprise at least one heterologous polynucleotide encoding a polypeptide having DHAD activity and at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis. For example, lactic acid bacteria comprising recombinant DHAD and at least one recombinant genetic expression element encoding Fe-S cluster forming proteins are the subject of U.S. Appl. No. 12/569,103, filed Sept. 29, 2009, which is incorporated by reference herein. The present recombinant host cells comprising at least one heterologous polynucleotide encoding a polypeptide having DHAD activity and at least one heterologous polynucleotide encoding a polypeptide field Sept. 29, 2009, which is incorporated by reference herein. The present recombinant host cells comprising the subject of use a polypeptide encoding a polypeptide field by not include those lactic acid bacteria described in U.S. Appl. No. 12/569,103, filed Sept. 29, 2009, which is incorporated by reference herein.
- [0130] The polypeptide affecting Fe-S cluster biosynthesis can be selected from the group consisting of the Fe uptake and utilization or Fe-S cluster biosynthesis pathway genes in Tables 7, 8 and 9. In one embodiment, the polypeptide affecting Fe-S cluster biosynthesis is encoded by ARN1, ARN2, ATX1, CCC2, COT1, ENB1, FET3, FET5, FIT1, FIT2, FIT3, FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FTH1, FTR1, HMX1, SIT1, SMF3, TIS11, VHT1, AFT1, AFT2, AIMI, ARHI, ATMI, BUD32, CADI, CCCI, CFDI, CIAI, CMKI, CTHI, CTI6, CYC8, DAPI, DRE2, ERV1, ESA1, FET4, FRA1, FRA2, GEF1, GGC1, GRX1, GRX2, GRX4, GRX5, HDA1, IBA57, ISA1, ISA2, ISU1, ISU2, JAC1, MGE1, MRS3, MRS4, MSN5, NAR1, NFS1, NFU1, NHP6a, NHP6b, PSE1, SMF1, SNF1, SNF2, SNF3, SNF4, SSQ1, TIM12, TUP1, NP_011911.1, VPS41, YAP5, YFH1, YRA1, ZPR1, iscAnif, nifU, nifS, cysE1, cysE2, iscS, iscU, iscA, hscB, hscA, Fdx, sufS, sufE, cysE3, sufS2, iscA2, Nfu, nfuA, nfuV, nfu, sufA, sufB, sufC, sufD, sufE1, sufS2, or sufE2. In one embodiment, the polypeptide affecting Fe-S cluster biosynthesis is AFT1, AFT2, PSE1, FRA2, GRX3, or MSN5. In one embodiment, the polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, PSE1, FRA2, GRX3, MSN5, and combinations thereof. In one embodiment, the polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, PSE1, FRA2,

endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis.

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MSN5, and combinations thereof. In another embodiment, the polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, PSE1, FRA2, GRX3, MSN5, and combinations thereof, and the polypeptide affecting Fe-S cluster biosynthesis is encoded by a polynucleotide comprising a plasmid. In some embodiments, DHAD is co-expressed with AFT1, AFT2, PSE1, and combinations thereof. The polypeptide affecting Fe-S cluster biosynthesis may be a constitutive mutant, such as, but not limited to, AFT1 L99A, AFT1 L102A, AFT1 C291F, AFT1 C293F, and combinations thereof. The deletion, mutation, and/or substitution in the endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis can be selected from the group consisting of FRA2, GRX3, MSN5, and combinations thereof.

[0131] The present invention also provides a method for increasing the activity of an Fe-S cluster requiring protein in a recombinant host cell comprising providing a recombinant host cell comprising an Fe-S cluster requiring protein, changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis in the host cell, and growing the recombinant host cell with the changed expression or activity under conditions whereby the activity of the Fe-S cluster requiring protein is increased. Such a method can be used to increase the activity of an endogenous Fe-S cluster requiring protein, or a heterologous Fe-S cluster requiring protein. Such a method can be used to increase the specific activity of a DHAD described herein, or identified by the methods described herein. The increase in the activity of the Fe-S cluster requiring protein can be in an amount selected from greater than about 10%; greater than about 15%; greater than about 20%; greater than about 25%; greater than about 30%; greater than about 35%; greater than about 40%; greater than about 45%; greater than about 50%; greater than about 55%; greater than about 60%; greater than about 65%; greater than about 70%; greater than about 75%; greater than about 80%; greater than about 85%; greater than about 90%; and greater than about 95%. The increase in activity may be greater than about 3 fold, greater than about 5 fold, greater than about 8 fold, or greater than about 10 fold. In embodiments, the activity of the Fe-S cluster requiring protein can be in an amount that is at least about 60% of theoretical, at least about 70% of theoretical, at least about 80% theoretical, or at least about 90% theoretical.

[0132] The present invention can also be used to increase the flux in the Fe-S cluster biosynthesis pathway in a host cell and to identify polypeptides that increase the flux in

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an Fe-S cluster biosynthesis pathway in a host cell. In one embodiment a method is provided for increasing the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising providing a recombinant host cell comprising an Fe-S cluster requiring protein and either at least one polypeptide affecting Fe-S cluster biosynthesis, at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis, or a combination of both, and growing the recombinant host cell under conditions whereby the flux in the Fe-S cluster biosynthesis pathway in the host cell is increased. In another embodiment, a method is provided for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising: (a) changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis; (b) measuring the activity of a Fe-S cluster requiring protein; and (c) comparing the activity of the Fe-S cluster requiring protein measured in the presence of the change in expression or activity polypeptide of step (a) to the activity of the Fe-S cluster requiring protein measured in the absence of the change in expression or activity polypeptide of step (a), wherein an increase in the activity of the heterologous Fe-S cluster requiring protein indicates an increase in the flux in said Fe-S cluster biosynthesis pathway. In such methods, the Fe-S cluster requiring protein may be endogenous or heterologous to the host cell.

- **[0133]** The expression or activity of the polypeptide affecting Fe-S cluster biosynthesis can be changed by methods well known in the art, including, but not limited to, deleting, mutating, substituting, expressing, up-regulating, down-regulating, altering the cellular location, altering the state of the protein, and/or adding a cofactor, and combinations thereof. Altering the state of the protein can include, but are not limited to, such alterations as phosphorylation or ubiquitination. Any number of methods described herein or known in the art can be used to measure the activity of the Fe-S cluster requiring protein, depending upon the Fe-S cluster requiring protein chosen. For example, if DHAD is the Fe-S cluster requiring protein, the assay described in the Example 7 can be used to measure the activity of the DHAD to determine if there is an increase in the flux in the Fe-S cluster biosynthesis pathyway of the host cell.
- [0134] Isobutanol and Other Products
- [0135] Expression of a DHAD in a recombinant host cell, as described herein, provides the transformed, recombinant host cell with dihydroxy-acid dehydratase activity for

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conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate or 2,3dihydroxymethylvalerate to α -ketomethylvalerate. A product that has α -ketoisovalerate or α -ketomethylvalerate as a pathway intermediate may be produced with greater effectiveness in a host cell disclosed herein having the described heterologous DHAD. A list of such products includes, but is not limited to, valine, isoleucine, leucine, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, and isobutanol.

- For example, biosynthesis of valine in yeast includes steps of acetolactate [0136] conversion to 2,3-dihydroxy-isovalerate by acetohydroxyacid reductoisomerase (ILV5), conversion of 2,3-dihydroxy-isovalerate to α -ketoisovalerate (also called 2-ketoisovalerate) by dihydroxy-acid dehydratase, and conversion of α -ketoisovalerate to valine by branched-chain amino acid transaminase (BAT2) and branched-chain animo acid Biosynthesis of leucine includes the same steps to α aminotransferase (BAT1). ketoisovalerate, followed by conversion of α -ketoisovalerate to alpha-isopropylmalate by alpha-isopropylmalate synthase (LEU9, LEU4), conversion of alpha-isopropylmalate to beta-isopropylmalate by isopropylmalate isomerase (LEU1), conversion of betaisopropylmalate to alpha-ketoisocaproate by beta-IPM dehydrogenase (LEU2), and finally conversion of alpha-ketoisocaproate to leucine by branched-chain amino acid transaminase (BAT2) and branched-chain amino acid aminotransferase (BAT1). The bacterial pathway is similar, involving differently named proteins and genes. Increased conversion of 2,3-dihydroxy-isovalerate to α -ketoisovalerate will increase flow in these pathways, particularly if one or more additional enzymes of a pathway is overexpressed. Thus, it is desired for production of valine or leucine to use a strain disclosed herein.
- [0137] Biosynthesis of pantothenic acid includes a step performed by DHAD, as well as steps performed by ketopantoate hydroxymethyltransferase and pantothenate synthase. Engineering of expression of these enzymes for enhanced production of pantothenic acid biosynthesis in microorganisms is described in U.S. Patent No. 6,177,264.
- [0138] The α -ketoisovalerate product of DHAD is an intermediate in isobutanol biosynthetic pathways disclosed in U.S. Patent Appl. Pub. No. 20070092957 A1, which is incorporated by reference herein. A diagram of disclosed isobutanol biosynthetic pathways is provided in Figure 5. Production of isobutanol in a strain disclosed herein may benefit from increased DHAD activity. As disclosed herein, increased DHAD activity is provided by expression of a DHAD in a host cell, for example, by over-

expressing the DHAD, by modulating the expression or activity of a polypeptide having Fe-S cluster regulatory activity, or a combination of both expression of a DHAD and modulation of the expression or activity of a polypeptide having Fe-S cluster regulatory activity. As described in U.S. Patent Appl. Pub. No. 20070092957 A1, which is incorporated by reference herein, steps in an example isobutanol biosynthetic pathway include conversion of:

- [0139] pyruvate to acetolactate (*see* Fig. 5, pathway step a therein), as catalyzed for example by acetolactate synthase,
- [0140] acetolactate to 2,3-dihydroxyisovalerate (*see* Fig. 5, pathway step b therein) as catalyzed for example by acetohydroxy acid isomeroreductase;
- [0141] 2,3-dihydroxyisovalerate to α-ketoisovalerate (*see* Fig. 5, pathway step c therein) as catalyzed for example by acetohydroxy acid dehydratase, also called dihydroxy-acid dehydratase (DHAD);
- [0142] α -ketoisovalerate to isobutyraldehyde (*see* Fig. 5, pathway step d therein) as catalyzed for example by branched-chain α -keto acid decarboxylase; and
- [0143] isobutyraldehyde to isobutanol (*see* Fig. 5, pathway step e therein) as catalyzed for example by branched-chain alcohol dehydrogenase.
- [0144] The substrate to product conversions, and enzymes involved in these reactions, for steps f, g, h, I, j, and k of alternative pathways are described in U.S. Patent Appl. Pub. No. 20070092957 A1, which is incorporated by reference herein.
- [0145] Genes that can be used for expression of the pathway step enzymes named above other than the DHADs disclosed herein, as well as those for additional isobutanol pathways, are described in U.S. Patent Appl. Pub. No. 20070092957 A1, which is incorporated by reference herein. Additional genes that may be used can be identified by one skilled in the art through bioinformatics or using methods well-known in the art, such as the various methods described in U.S. Appl. No. 12/569,636, filed Sept. 29, 2009, which is incorporated by reference herein, to isolate homologs. Suitable ketol-acid reductoisomerase (KARI) enzymes are described in U.S. Appl. No. 12/893077, all incorporated by reference herein. Examples of KARIs disclosed therein are those from *Vibrio cholerae, Pseudomonas aeruginosa* PAO1, and *Pseudomonas fluorescens* PF5.

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U.S. Patent Appl. Publ. No. 2009/0269823 and U.S. Prov. Patent Appl. No. 61/290,636, incorporated by reference herein, describe suitable alcohol dehydrogenases.

- [0146] Additionally described in U.S. Patent Appl. Pub. No. 20070092957 A1, which is incorporated by reference herein, are construction of chimeric genes and genetic engineering of bacteria and yeast for isobutanol production using the disclosed biosynthetic pathways.
- [0147] Additional modifications
- [0148]
- [0149] Examples of additional modifications that may be useful in cells provided herein include modifications to reduce glycerol-3-phosphate dehydrogenase activity and/or disruption in at least one gene encoding a polypeptide having pyruvate decarboxylase activity or a disruption in at least one gene encoding a regulatory element controlling pyruvate decarboxylase gene expression as described in U.S. Patent Appl. Pub. No. 20090305363 (incorporated herein by reference), modifications to a host cell that provide for increased carbon flux through an Entner-Doudoroff Pathway or reducing equivalents balance as described in U.S. Patent Appl. Pub. No. 20100120105 (incorporated herein by Other modifications include integration of at least one polynucleotide reference). encoding a polypeptide that catalyzes a step in a pyruvate-utilizing biosynthetic pathway described in U.S. Prov. Appl. No. 61/380563 (incorporated herein by reference). Additional modifications that may be suitable are described in U.S, Appl. Serial No. 12/893089. Additionally, host cells comprising a heterologous polynucleotide encoding a polypeptide with phosphoketolase activity and host cells comprising a heterologous polynucleotide encoding a polypeptide with phosphotransacetylase activity are described in US Provisional Patent Application No. 61/356379. Growth for production
- [0150] Recombinant host cells disclosed herein are grown in fermentation media which contains suitable carbon substrates. Suitable carbon substrates may include, but are not limited to, monosaccharides such as glucose, fructose, oligosaccharides such as lactose maltose, galactose, or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Other carbon substrates may include ethanol, lactate, succinate, or glycerol.

- [0151] Additionally the carbon substrate may also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. Two-carbon substrates such as ethanol may also suitable. In addition to one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeasts are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion *et al., Microb. Growth Cl Compd.*, [Int. Symp.], 7th (1993), 415-32, Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter *et al., Arch. Microbiol.* 153:485-489 (1990)). Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism.
- [0152] Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, in some embodiments, the carbon substrates are glucose, fructose, and sucrose, or mixtures of these with C5 sugars such as xylose and/or arabinose for yeasts cells modified to use C5 sugars. Sucrose may be derived from renewable sugar sources such as sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof. Glucose and dextrose may be derived from renewable grain sources through saccharification of starch based feedstocks including grains such as corn, wheat, rye, barley, oats, and mixtures thereof. In addition, fermentable sugars may be derived from renewable cellulosic or lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in co-owned and co-pending U.S. Patent Appl. Pub. No. 20070031918 A1, which is herein incorporated by reference. Biomass refers to any cellulosic or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Biomass may also comprise additional components, such as protein and/or lipid. Biomass may be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example, biomass may comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste,

wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

- [0153] In addition to an appropriate carbon source, growth media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of an enzymatic pathway comprising a Fe-S cluster requiring protein such as, for example, DHAD.
- [0154] <u>Culture Conditions</u>
- [0155] Typically cells are grown at a temperature in the range of about 20 °C to about 40 °C in an appropriate medium. Suitable growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, Yeast Medium (YM) broth, or broth that includes yeast nitrogen base, ammonium sulfate, and dextrose (as the carbon/energy source) or YPD Medium, a blend of peptone, yeast extract, and dextrose in optimal proportions for growing most *Saccharomyces cerevisiae* strains. Other defined or synthetic growth media may also be used, and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, *e.g.*, cyclic adenosine 2':3'-monophosphate, may also be incorporated into the growth medium.
- [0156] Suitable pH ranges for the growth are between about pH 5.0 to about pH 9.0. In one embodiment, about pH 6.0 to about pH 8.0 is used for the initial condition. Suitable pH ranges for the fermentation of yeast are typically between about pH 3.0 to about pH 9.0. In one embodiment, about pH 5.0 to about pH 8.0 is used for the initial condition. Suitable pH ranges for the fermentation of other microorganisms are between about pH 3.0 to about pH 3.0 to about pH 7.5. In one embodiment, about pH 4.5 to about pH 6.5 is used for the initial condition.
- [0157] Growth may be performed under aerobic or anaerobic conditions. In one embodiment, anaerobic or microaerobic conditions are used for growth.
- [0158] Industrial Batch and Continuous Fermentations

- [0159] Isobutanol, or other products, may be produced using a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. A variation on the standard batch system is the fed-batch system. Fed-batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the media. Batch and fed-batch fermentations are common and well known in the art and examples may be found in Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227, (1992), herein incorporated by reference.
- **[0160]** Isobutanol, or other products, may also be produced using continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth. Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.
- [0161] It is contemplated that the production of isobutanol, or other products, may be practiced using batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isobutanol production.
- [0162] <u>Methods for Isobutanol Isolation from the Fermentation Medium</u>
- [0163] Bioproduced isobutanol may be isolated from the fermentation medium using methods known in the art for ABE fermentations (*see, e.g.*, Durre, *Appl. Microbiol. Biotechnol.* 49:639-648 (1998), Groot *et al.*, *Process. Biochem.* 27:61-75 (1992), and

references therein). For example, solids may be removed from the fermentation medium by centrifugation, filtration, decantation, or the like. Then, the isobutanol may be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation.

- **[0164]** Because isobutanol forms a low boiling point, azeotropic mixture with water, distillation can be used to separate the mixture up to its azeotropic composition. Distillation may be used in combination with another separation method to obtain separation around the azeotrope. Methods that may be used in combination with distillation to isolate and purify butanol include, but are not limited to, decantation, liquid-liquid extraction, adsorption, and membrane-based techniques. Additionally, butanol may be isolated using azeotropic distillation using an entrainer (*see, e.g.*, Doherty and Malone, *Conceptual Design of Distillation Systems*, McGraw Hill, New York, 2001).
- **[0165]** The butanol-water mixture forms a heterogeneous azeotrope so that distillation may be used in combination with decantation to isolate and purify the isobutanol. In this method, the isobutanol containing fermentation broth is distilled to near the azeotropic composition. Then, the azeotropic mixture is condensed, and the isobutanol is separated from the fermentation medium by decantation. The decanted aqueous phase may be returned to the first distillation column as reflux. The isobutanol-rich decanted organic phase may be further purified by distillation in a second distillation column.
- [0166] The isobutanol may also be isolated from the fermentation medium using liquidliquid extraction in combination with distillation. In this method, the isobutanol is extracted from the fermentation broth using liquid-liquid extraction with a suitable solvent. The isobutanol-containing organic phase is then distilled to separate the butanol from the solvent.
- [0167] Distillation in combination with adsorption may also be used to isolate isobutanol from the fermentation medium. In this method, the fermentation broth containing the isobutanol is distilled to near the azeotropic composition and then the remaining water is removed by use of an adsorbent, such as molecular sieves (Aden *et al. Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, Report NREL/TP-510-32438, National Renewable Energy Laboratory, June 2002).

[0168] Additionally, distillation in combination with pervaporation may be used to isolate and purify the isobutanol from the fermentation medium. In this method, the fermentation broth containing the isobutanol is distilled to near the azeotropic composition, and then the remaining water is removed by pervaporation through a hydrophilic membrane (Guo *et al.*, *J. Membr. Sci.* 245, 199-210 (2004)).

[0169] <u>Embodiments of the Inventions</u>

- [0170] Embodiment 1 (E1). A recombinant host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity wherein said at least one heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated.
- [0171] E2. A recombinant host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity wherein said at least one heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.
- [0172] E3. A recombinant host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity, wherein said host cell comprises at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis.
- [0173] E4. A recombinant host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity and at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis.
- [0174] E5. The recombinant host cell of any one of embodiments E3-E4, wherein said heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of the genes in Tables 8 and 9.
- [0175] E6. The recombinant host cell of any one of embodiments E3-E4, wherein said heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of the genes in Table 7.
- [0176] E7. The recombinant host cell of embodiment E5 or E6, wherein said heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is

selected from the group consisting of AFT1, AFT2, PSE1, FRA2, GRX3, MSN5. and combinations thereof.

- [0177] E8. The recombinant host cell of embodiment E7, wherein said polypeptide is encoded by a polynucleotide that is constitutive mutant.
- [0178] E9. The recombinant host cell of embodiment E8, wherein said constitutive mutant is selected from the group consisting of AFT1 L99A, AFT1 L102A, AFT1 C291F, AFT1 C293F, and combinations thereof.
- **[0179]** E10. The recombinant host cell of embodiment E7, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by a polynucleotide comprising a high copy number plasmid or a plasmid with a copy number that can be regulated.
- [0180] E11. The recombinant host cell of embodiment E7, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by a polynucleotide integrated at least once in the recombinant host cell DNA.
- [0181] E12. The recombinant host cell of embodiment E3, wherein the at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of FRA2, GRX3, MSN5, and combinations thereof.
- [0182] E13. The recombinant host cell of embodiment E4, wherein the at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, PSE1, and combinations thereof.
- **[0183]** E14. The recombinant host cell of any one of embodiments E3-E13, wherein said at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is expressed in multiple copies.
- [0184] E15. The recombinant host cell of embodiment E14, wherein said at least one heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated.
- [0185] E16. The recombinant host cell of embodiment E14, wherein said at least one heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.
- [0186] E17. The recombinant host cell of any one of embodiments E3-E16, wherein said Fe-S cluster biosynthesis is increased compared to a recombinant host cell having endogenous Fe-S cluster biosynthesis.

- [0187] E18. The recombinant host cell of any one of embodiments E1-E17, wherein said host cell is a yeast host cell.
- [0188] E19. The recombinant host cell of embodiment E18, wherein said yeast host cell is selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia* and *Pichia*.
- [0189] E20. The recombinant host cell of any one of embodiments E1-E19, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in the cytosol of the host cell.
- [0190] E21. The recombinant host cell of any one of embodiments E1-E20, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of $< 10^{-5}$ wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the *Streptococcus mutans* DHAD enzyme corresponding to SEQ ID NO:168.
- [0191] E22. The recombinant host cell of any one of embodiments E1-E21, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence with at least about 90% identity to SEQ ID NO: 168 or SEQ ID NO: 232.
- [0192] E23. The recombinant host cell of any one of embodiments E1-E22, wherein said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of:
 - a. greater than about 5-fold with respect to the control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity;
 - b. greater than about 8-fold with respect to the control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and
 - c. greater than about 10-fold with respect to the control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity.
- [0193] E24. The recombinant host cell of any one of embodiments E1-E22, wherein said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of:

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- a. greater than about 0.25 U/mg;
- b. greater than about 0.3 U/mg;
- c. greater than about 0.5 U/mg;
- d. greater than about 1.0 U/mg;
- e. greater than about 1.5 U/mg;
- f. greater than about 2.0 U/mg;
- g. greater than about 3.0 U/mg;
- h. greater than about 4.0 U/mg;
- i. greater than about 5.0 U/mg;
- j. greater than about 6.0 U/mg;
- k. greater than about 7.0 U/mg;
- 1. greater than about 8.0 U/mg;
- m. greater than about 9.0 U/mg;
- n. greater than about 10.0 U/mg;
- o. greater than about 20.0 U/mg; and
- p. greater than about 50.0 U/mg.
- [0194] E25. The recombinant host cell of any one of embodiments E1-E24, wherein said recombinant host cell produces isobutanol.
- [0195] E26. The recombinant host cell of embodiment E25, wherein said recombinant host cell comprises an isobutanol biosynthetic pathway.
- [0196] E27. A method of making a product comprising:
 - a. providing the recombinant host cell of any one of embodiments E1-E24; and

b. contacting the recombinant host cell of (a) with a fermentable carbon substrate in a fermentation medium under conditions wherein said product is produced;

wherein the product is selected from the group consisting of branched chain amino acids, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol, and combinations thereof.

- [0197] E28. A method of making isobutanol comprising:
 - a. providing the recombinant host cell of any one of embodiments E1-E24;
 - b. contacting the recombinant host cell of (a) with a fermentable carbon substrate in a fermentation medium under conditions wherein isobutanol is produced.
- [0198] E29. A method for the conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate comprising:
 - a. providing the recombinant host of any one of embodiments E1-E24;
 - b. growing the recombinant host cell of (a) under conditions where the 2,3dihydroxyisovalerate is converted to α -ketoisovalerate,

wherein 2,3-dihydroxyisovalerate is converted to α -ketoisovalerate.

- [0199] E30. A method for increasing the specific activity of a heterologous polypeptide having dihydroxy-acid dehydratase activity in a recombinant host cell comprising:
 - a. providing a recombinant host cell of any one of embodiments E1-E24; and
 - b. growing the recombinant host cell of (a) under conditions whereby the heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in functional form having a specific activity greater than the same host cell lacking said heterologous polypeptide.
- [0200] E31. A method for increasing the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising:
 - a. providing a recombinant host cell of any one of embodiments E3-E24; and

- b. growing the recombinant host cell of (a) under conditions whereby the flux in the Fe-S cluster biosynthesis pathway in the host cell is increased.
- [0201] E32. A method of increasing the activity of an Fe-S cluster requiring protein in a recombinant host cell comprising:
 - a. providing a recombinant host cell comprising an Fe-S cluster requiring protein;
 - b. changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis in said host cell; and
 - c. growing the recombinant host cell of (b) under conditions whereby the activity of the Fe-S cluster requiring protein is increased.
- [0202] E33. The method of embodiment E32, wherein said increase in activity is an amount selected from the group consisting of:
 - a. greater than about 10%;
 - b. greater than about 20%;
 - c. greater than about 30%;
 - d. greater than about 40%;
 - e. greater than about 50%;
 - f. greater than about 60%;
 - g. greater than about 70%;
 - h. greater than about 80%;
 - i. greater than about 90%; and
 - j. greater than about 95%.
- [0203] E34. The method of embodiment E32, wherein said increase in activity is an amount selected from the group consisting of:
 - a. greater than about 5 fold;

- b. greater than about 8 fold;
- c. greater than about 10 fold.
- [0204] E35. A method for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising:
 - a. changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis;
 - b. measuring the activity of a heterologous Fe-S cluster requiring protein; and
 - c. comparing the activity of the heterologous Fe-S cluster requiring protein measured in the presence of the changed expression or activity of a polypeptide of step (a) to the activity of the heterologous Fe-S cluster requiring protein measured in the absence of the changed expression or activity of a polypeptide of step (a),

wherein an increase in the activity of the heterologous Fe-S cluster requiring protein indicates an increase in the flux in said Fe-S cluster biosynthesis pathway.

- [0205] E36. A method for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising:
 - a. changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis;
 - b. measuring the activity of a polypeptide having dihydroxy-acid dehydratase activity; and
 - c. comparing the activity of the polypeptide having dihydroxy-acid dehydratase activity measured in the presence of the change in expression or activity of a polypeptide of step (a) to the activity of the polypeptide having dihydroxy-acid dehydratase activity measured in the absence of the change in expression or activity of a polypeptide of step (a),

wherein an increase in the activity of the polypeptide having dihydroxy-acid dehydratase activity indicates an increase in the flux in said Fe-S cluster biosynthesis pathway.

- [0206] E37. The method of any one of embodiments E30-E36, wherein said changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis comprises deleting, mutating, substituting, expressing, up-regulating, down-regulating, altering the cellular location, altering the state of the protein, and/or adding a cofactor.
- [0207] E38. The method of any one of embodiments E32-E37, wherein the Fe-S cluster requiring protein has dihydroxy-acid dehydratase activity and wherein said Fe-S cluster requiring protein having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of $< 10^{-5}$ wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the *Streptococcus mutans* DHAD enzyme corresponding to SEQ ID NO:168.
- [0208] E39. The method of any one of embodiments E32-E38, wherein said polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of the genes in Tables 7, 8 and 9.
- [0209] E40. A recombinant host cell comprising at least one polynucleotide encoding a polypeptide identified by the methods of any one of embodiments E35-E37.
- **[0210]** E41. The recombinant host cell of embodiment E40, wherein said host cell further comprises at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity.
- [0211] E42. The recombinant host cell of embodiment E41, wherein said heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is expressed in multiple copies.
- [0212] E43. The recombinant host cell of embodiment E41, wherein said heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated.
- [0213] E44. The recombinant host cell of embodiment E41, wherein said heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.
- [0214] E45. The method of embodiment E35 or E36, wherein said host cell is a yeast host cell.
- [0215] E46. The method of embodiment E45, wherein said yeast host cell is selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia*, and *Pichia*.

- [0216] E47. The method of any one of embodiments E28-E39, wherein said host cell is a yeast host cell.
- [0217] E48. The method of embodiment E47, wherein said yeast host cell is selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia*, and *Pichia*.
- [0218] E49. The recombinant host cell of any one of embodiments E40-E44, wherein said recombinant host cell is a yeast host cell.
- [0219] E50. The recombinant host cell of embodiment E49, wherein said yeast host cell is selected from the group consisting of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia*, and *Pichia*.
- [0220] E51. The recombinant host cell of any one of embodiments E40-E44 or E49-E50, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in the cytosol of the host cell.
- [0221] E52. The recombinant host cell of any one of embodiments E40-E44 or E49-E50, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of $< 10^{-5}$ wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the *Streptococcus mutans* DHAD enzyme corresponding to SEQ ID NO:168.
- [0222] E53. The recombinant host cell of any one of embodiments E40-E44 or E49-E50, wherein said recombinant host cell produces a product selected from the group consisting of branched chain amino acids, pantothenic acid, 2-methyl-1-butanol, 3methyl-1-butanol, isobutanol, and combinations thereof.
- [0223] E54. The recombinant host cell of embodiment E53, wherein said recombinant host cell produces isobutanol.
- [0224] E55. The recombinant host cell of embodiment E54, wherein said recombinant host cell comprises an isobutanol biosynthetic pathway.

Examples

[0225] The meaning of abbreviations used is as follows: "min" means minute(s), "h" means hour(s), "sec" means second(s), "µl" means microliter(s), "ml" means milliliter(s), "L" means liter(s), "nm" means nanometer(s), "mm" means millimeter(s), "cm" means

centimeter(s), " μ m" means micrometer(s), "mM" means millimolar, "M" means molar, "mmol" means millimole(s), " μ mole" means micromole(s), "g" means gram(s), " μ g" means microgram(s), "mg" means milligram(s), "rpm" means revolutions per minute, "w/v" means weight/volume, "OD" means optical density, and "OD₆₀₀" means optical density measured at a wavelength of 600 nm.

[0226] GENERAL METHODS:

- [0227] Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984, and by Ausubel, F. M. *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience, N.Y., 1987.
- [0228] Materials and methods suitable for the maintenance and growth of bacterial cultures are also well known in the art. Techniques suitable for use in the following Examples may be found in *Manual of Methods for General Bacteriology*, Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, DC., 1994, or by Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, MA, 1989. All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), BD Diagnostic Systems (Sparks, MD), Life Technologies (Rockville, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified.

[0229] Example 1. Over-expression of DHAD protein encoded by the *ilvD* gene from *S. mutans* using a plasmid-based system in yeast cytosol.

[0230] Over-expression of a recombinant polynucleotide can be accomplished by increasing the copy number of a plasmid comprising the recombinant polynucleotide. To over-express the DHAD protein in yeast, an inducible vector was constructed. The pHR81 vector contains a *Ura3* marker as well as a *LEU* marker with a defective promoter

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(*see* U.S. Patent Appl. Pub. No. 2007/0092957, which is incorporated by reference herein). When the yeast synthetic dropout (SD; also known as complete minimal media; Teknova) growth medium is switched from SD minus uracil to SD minus leucine, the copy number of the pHR81 plasmid increases, resulting in much higher level of expression of the recombinant polynucleotide. The pHR81 vector backbone was derived from pLH472 JEG4y (SEQ ID NO: 921) and was prepared by digesting the pLH472 JEG4y vector with SpeI and SacII.

- [0231] For over-expression of a DHAD protein, the DHAD gene *ilvD* from *S. mutans* (SEQ ID NO:167) was used (*see* U.S. Published Patent Appl. No. US2009-0305363A1, which is incorporated by reference herein). This gene has been cloned under the control of the FBA promoter in vector pRS423 FBA ilvD Strep-lumio (*see* U.S. Published Patent Appl. No. US2009-0305363A1, which is incorporated by reference herein). The region containing the FBA promoter, the *ilvD* gene, and FBA terminator cassette was amplified with primer set FBAp-F(NheI) and FBAt-R(SacII) (SEQ ID NOs: 915 and 916) and cloned into the pHR81 vector. The resulting expression vector was designated as pHR81 FBA-IlvD(Sm) (SEQ ID NO: 917; Figure 1A).
- [0232] To over express the *S. mutans* DHAD protein, the expression vector pHR81 FBA-IlvD(Sm) was transformed into wild-type yeast strain BY4741. Transformants were selected on agar plates with SD minus uracil. For over-expression, yeast strains containing the plasmid were initially grown at 30° C in SD liquid medium minus uracil. A fresh overnight culture (5 ml) was then transferred to a 125 ml flask containing 75 ml of SD medium minus leucine. As a control, another 5 ml of fresh overnight culture was transferred into a flask containing 75 ml of SD minus uracil. The cultures were incubated overnight before harvesting by centrifugation. The DHAD activity was measured in crude extracts of these samples using the assay described in Example 7.
- [0233] The DHAD specific activity obtained in the crude extract in the control samples grown in SD minus uracil was in the range of 0.2 to 0.3 U mg⁻¹. The average specific activity obtained from strains grown in the SD medium minus leucine, however, was 1.6 U mg⁻¹, much higher (~5 to 8-fold higher) than the activity from the control samples. DHAD requires Fe-S cluster for its function, and it was not previously known if the native yeast Fe-S cluster biosynthesis pathway could accommodate an over-expressed Fe-S cluster requiring protein in yeast cytosol. In a previous screening experiment using a

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non-inducible, low-copy number vector, the DHAD from *S. mutans* could be recombinantly expressed in yeast cytosol with a specific activity in the range of 0.1 to 0.2 U mg⁻¹ in the crude extract (*see* U.S. Patent Appl. No. 12/569,636, filed on Sept. 29, 2009, which is incorporated by reference herein). Thus, in one embodiment, over-expression of a Fe-S cluster requiring protein, such as DHAD, in yeast using a high-copy number vector provides increased specific activity, wherein the specific activity is increased by at least about 5-fold to at least about 8-fold.

[0234] Example 2. Over-expression of DHAD protein encoded by the *ilvD* gene from *S. mutans* through chromosomal integration.

- [0235] An alternate way to increase the expression of a gene in yeast is to integrate multiple copies of the gene of interest into the host cell's chromosome. To integrate the *ilvD* gene from *S. mutans* (SEQ ID NO:167) into a yeast chromosome, integration vector pZK-Delta(s)-Leu2-FBA-ilvD(Sm)-FBAt (SEQ ID NO: 918; Figure 1B) was constructed. The integration vector backbone was derived from pSuperscript (Stratagene, La Jolla, CA). The *S. mutans ilvD* gene (nucleotides 1306-3018 of the complement strand) was cloned into the integration vector under the control of the FBA promoter (nucleotides 3026-4023 of the complement strand) so that the *ilvD* gene would be flanked by a yeast delta sequence (nucleotides 118-267 and 5061-5760 of the complement strand). *S. cerevisiae* contains more than 200 yeast delta sequences (Kim J M *et al.* Genome Res. 1998; 8:464-478). These delta sequences are targets for multiple integrations. The integration vector was also engineered to contain the defective *LEU2* marker (nucleotides 4100-5191 of the complement strand) for selection of transformed strains with multiple integration events.
- [0236] For integration, the vector DNA was linearized with AscI and AatII digestion to generate delta sequence flanked strands of vector DNA comprising the *ilvD* gene, which were then transformed into the yeast strain BY4741. Transformants were selected on SD agar medium minus leucine. These transformants were then grown on SD liquid medium minus leucine at 30° C, and the cultures were harvested and analyzed for DHAD activity. The specific activity of DHAD obtained in the crude extract ranged from 0.7 to 1.2 U

 mg^{-1} . This specific activity was about 3- to 6-fold higher than that found in BY4741 strains transformed with an *ilvD* gene-containing plasmid without over-expression

[0237] Example 3. Improvement of specific activity of DHAD in yeast deletion strains

- **[0238]** Although the over-expression strains described in Examples 1 and 2 had a high level of activity, not all of the DHAD protein expressed was active. For example, the over-expressed DHAD protein accounted for approximately 5 to 10% of the total cell protein, while yielding a specific activity of from about 0.7 to 1.6 U mg⁻¹. Given that the specific activity of the purified DHAD enzyme from *S. mutans* is 100 U mg⁻¹, expression of DHAD at 10% of total cell protein would be expected to yield a specific activity upwards of 5 to 10 U mg⁻¹. Although not wishing to be bound by one theory, the difference between the expected and observed specific activity was likely a result of insufficient Fe-S cluster loading. Thus, increasing Fe-S cluster loading by further manipulating the over-expression strains could be used to increase the specific activity of DHAD.
- **[0239]** In order to improve the specific activity, yeast strains with deletions in genes involved in iron metabolism and Fe-S cluster sensing were used to investigate their effects on DHAD specific activity. These strains (BY4741 background) were purchased from Open Biosystem (Huntsville, AL) and are listed in Table 10. As described in Example 1, the high copy number plasmid pHR81 FBA-IlvD(Sm) was transformed into these strains, and DHAD over-expression was induced by changing the growth medium to SD minus leucine. Crude extracts from cultures were prepared and assayed for DHAD activity. Results are shown in Table 10.

Genes	Function	Specific Activity (U/mg)
WT		1.69 ± 0.02
$\Delta isul$	scaffold protein for Fe-S cluster assembling	1.31 ± 0.56
$\Delta fra2$	repressor component for Aft1p	3.41 ± 0.24
$\Delta sin4$	regulatory protein	1.65 ± 0.20
$\Delta m tm l$	protein involved in metal metabolism	0.54 ± 0.12
Δ fra1	regulatory protein	0.97 ± 0.05
$\Delta grx3$	glutaredoxins	5.45 ± 0.14

Table 10. Effects of deletions of genes involved in Fe metabolism.

$\Delta a ft l$	global Fe regulator	0.23 ± 0.05
$\Delta aft2$	paralogue to Aft1p	1.11 ± 0.38
$\Delta msn5$	nuclear protein exporter	1.59 ± 0.10
$\Delta fet3$	ferrous iron uptake; multi-copper oxidase	0.54 ± 0.09
$\Delta ftr 1$	ferrous iron uptake; permease	0.76 ± 0.03
$\Delta ccc2$	copper transporter (for Fet3p)	1.23 ± 0.17
$\Delta gefl$	copper transporter/loading for Fet3p	1.70 ± 0.10
$\Delta fet 4$	Low-affinity Fe(II) transporter	1.07 ± 0.02
$\Delta smfl$	Low-affinity Fe(II) transporter	1.78 ± 0.12
$\Delta mrs3$	mitochondrial iron transporter	1.51 ± 0.13
$\Delta mrs4$	mitochondrial iron transporter	0.85 ± 0.16
$\Delta cth2$	targeted mRNA binding and degradation	1.28 ± 0.40
$\Delta cth1$	targeted mRNA binding and degradation 1.44 ± 0.30	

- [0240] Surprisingly, DHAD specific activity in the crude extract in strains with a deletion in either the *FRA2* or the *GRX3* gene increased by 2- to 3-fold, which was unexpected as many of the deletions tested did not increase DHAD specific activity. It has been shown that cytosolic iron sulfur assembly (CIA) machinery in yeast is responsible for assembly of Fe-S clusters for cytosolic proteins such as isopropylmalate isomerase (Leu1). Previous results indicate that this CIA machinery is independent from the iron sensing system involving Aft1 and a Grx3/Grx4-Fra2 heterodimer as the repressor (Rutherford *et al, J Biol Chem. 280*:10135-10140 (2005)).
- [0241] Another unexpected finding is the effect of a *Grx3* deletion on DHAD activity. It has been shown that Grx3 and Grx4 are equivalent in function. While double mutations in both *GRX3* and *GRX4* genes resulted in drastic activation of the Fe regulon, mutation in Grx4 alone confers minimal phenotype (Pujol-Carrion, *et al*, *J Cell Sci. 119*:4554-4564 (2006); Ojeda, *et al*, *J Biol Chem. 281*:17661-17669 (2006).). As shown in Table 10 above, *GRX3* deletion alone leads to significant improvement in DHAD specific activity.
- [0242] Thus, these results demonstrate that modulating genes involved in iron metabolism can increase the activity of an Fe-S cluster requiring protein such as DHAD when expressed in yeast cytosol. As outlined in Figure 10, the effect of deletions of the *FRA2* and *GRX3* genes on DHAD specific activity could result from, *e.g.*, activation of transcription of one or more of the genes in the iron regulon via the global regulator

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Aft1p. Although not wishing to be bound by any one theory, activation of such genes could lead to an increase in iron uptake and an increase in cytoplasmic Fe-S cluster biosynthesis, leading to higher Fe-S cluster loading of the protein (Figure 10). Demonstration of increased Fe-S cluster loading is described in Example 11.

[0243] Example 4. Effect of expression of Aft1p and its mutants on DHAD specific activity.

- [0244] As described in Example 3 and outlined in Figure 10, Fra2, Grx3, and Grx4 are repressors that regulate the function of Aft1p (Kumánovics, *et al., J. Biol. Chem.* 283:10276-10286 (2008)). Aft1p is a global regulator of iron. Activation of genes involved in iron uptake and metabolism requires the nuclear localization of Aft1p. Expression of Aft1 constitutive mutants or an increase in the expression of wild-type Aft1p, could lead to the activation of the Fe regulon in a wild-type strain or in an *AFT1* deletion strain (Yamaguchi-Iwai, *et al, EMBO J. 14*:1231-1239 (1995); Yamaguchi-Iwai, *et al, J. Biol. Chem.* 277:18914-18918 (2002); Kaplan, *et al, Chem. Rev.109*:4536-4552(2009)). Based on the novel findings described in Example 3, it is possible that expression of Aft1p protein and its constitutive mutants may improve the active fraction of the DHAD enzyme which requires Fe-S clusters for its activity.
- **[0245]** To examine this possibility, the wild-type AFTI gene and its constitutive mutants were cloned using a centromere vector pRS411 (ATCC[®] Number: 87538; SEQ ID NO: 919). This vector has an ampicillin selection marker for growth in *E. coli* and a methionine nutritional marker for selection in yeast. The wild-type AFTI gene, including its own promoter and terminator, can be cloned between the KpnI and SacI sites, resulting in the construct pRS411-Aft1+flanking (SEQ ID NO: 920; Figure 2). A similar strategy can be used to clone genes that encode Aft1 constitutive mutants. The Aft1 constitutive mutants with substitutions at amino acids L99 to A and C291 to F (with respect to SEQ ID NO: 703) were first examined. The pRS411 constructs with genes encoding the wild-type AFTI gene or constitutive mutants were transformed, along with the expression vector pHR81 FBA IIvD(Sm), into the wild-type yeast strain BY4741 or a yeast strain with a deletion in AFTI, GRX3, or FRA2. Transformants were selected on agar plates with SD medium minus methionine and uracil. Transformed strains were grown in SD

medium minus methionine and leucine to over-express the DHAD protein in the presence of these genes or mutants. The DHAD activity in the crude extract of these cultures were measured.

[0246] Results of expression of wild-type Aft1p, Aft1p(C291F), and Aft1p(L99A) are shown in Table 11. A moderate increase in DHAD specific activity was observed with Aft1p (C291F) as compared to wild-type Aft1p. A much higher increase in DHAD activity was observed with Aft1p(L99A). The specific activity of DHAD in yeast expressing Aft1p(L99A) was similar to the specific activity obtained in the GRX3 deletion strain (*see* Table 10).

Table 11. Effects of expression of Aft1p and its mutants on the activity of DHAD from S. *mutans* in $\Delta aft1$ strain.

Plasmids	Specific Activity (U/mg)
pHR81-FBA-ilvD(Sm) + pRS411-Aft1	2.60 ± 0.52
pHR81-FBA-ilvD(Sm) + pRS411-Aft1(C291L)	3.79 ± 0.23
pHR81-FBA-ilvD(Sm) + pRS411-Aft1(L99A)	5.41 ± 0.41

[0247] Example 5. Increase in cytosolic DHAD specific activity in a CCC1 deletion strain.

[0248] The exact mechanism of increasing Fe-S cluster biosynthesis capability for cytosolic DHAD protein is unknown. Based on the findings with FRA2 and GRX3 deletion strains (Example 3) and with expression of Aft1p mutants (Example 4), increasing the availability of the Fe content in the cytosol may also improve the DHAD specific activity. CCC1 deletion has been shown to increase the Fe content of the cytosol (Li L, *et al*, *J Biol Chem. 276*:29515-29519 (2001)). To test this hypothesis, the CCC1 deletion strain of BY4741 was transformed with plasmid pHR81 FBA-IlvD(Sm) as described in Example 1. The crude extracts of cells with the plasmid were assayed for DHAD activity. Table 13 shows the results of the experiment. When the CCC1 deletion strain with the DHAD plasmid was grown in SD medium lacking uracil, an increase in DHAD specific activity was found as compared to the wild-type cells with the same plasmid. When extra Fe was added, a further increase in DHAD was observed in the

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CCC1 deletion strain. Addition of Fe showed no effect on DHAD specific activity in the wild-type cells. To achieve an over expression of the DHAD protein, strains were grown in SD medium lacking leucine (Example 1). Under these conditions, an increase in DHAD specific activity was detected.

Strains	Growth conditions	No extra Fe	100 uM Fe
Wild-type	-Ura	0.37 ± 0.03	0.46 ± 0.04
$\Delta ccc1$	-Ura	0.83 ± 0.04	1.24 ± 0.03
Wild-type	-Leu	1.60 ± 0.17	1.83 ± 0.31
$\Delta ccc1$	-Leu	2.53 ± 0.29	2.7 ± 1.07

Table 13. Expression of DHAD from S. mutans in the BY4741($\Delta ccc1$) strain.

[0249] Example 6. Improvement of specific activity of DHAD from *L. lactis* expressed in yeast.

[0250] Examples 1-5 used the DHAD enzyme from S. mutans to identify novel ways to increase the specific activity of DHAD when expressed in yeast. In this example, we investigated the application of these methods to improve the specific activity of the DHAD enzyme from L. lactis (SEQ ID NO: 958). The IlvD gene from L. lactis (SEQ ID NO: 959) was cloned into the pHR81 vector under the control of the FBA promoter (Figure 11). The resulting construct pHR81 FBA-IlvD(Ll)-ADHt (Figure 11; SEQ ID NO: 960) was transformed into strains with a deletion in either the FRA2 or GRX3 gene. To study the effect of the constitutive mutant Aft1p(L99A) on DHAD from L. lactis, pHR81 FBA-IlvD(Ll)-ADHt was co-transformed into yeast host along with vector pRS411-Aft1(L99A) (see Example 4). To over-express the IlvD gene, transformants were grown in yeast synthetic drop-out medium lacking leucine or lacking both leucine and methionine, depending on the strains. Enzymatic assay results are summarized in Table 14. Deletions in FRA2 and GRX3 genes increased the specific activity of the DHAD from L. lactis when expressed in yeast. In addition, expression of the Aft1 constitutive mutant L99A similarly increased the specific activity of the DHAD from L. lactis.

Table 14. Over-expression of bacterial DHAD from L. lactis in S. cerevisiae.

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Strains	Specific Activity (U/mg)
Wild-type	0.23 ± 0.04
$\Delta aft1 + Aft1(L99A)$	0.95 ± 0.31
$\Delta fra2$	0.72 ± 0.04
$\Delta grx3$	0.96 ± 0.05

[0251] Example 7. Determining the Specific Activity of DHAD. (Assay Method)

Quantitation of the activity of proteins requiring Fe-S clusters can be done in an [0252] assay format. If the protein is an enzyme, such as DHAD, the activity is typically expressed in terms of units of activity. A unit of enzyme activity has been defined by the Enzyme Commission of the International Union of Biochemistry as the amount of enzyme that will catalyze the transformation of 1 micromole of the substrate per minute under standard conditions (International Union of Biochemistry, Report of the Commission on Enzymes, Oxford: Pergamon Press, 1961). Further, the term specific activity is defined as the units of activity in a given amount of enzyme. Thus, the specific activity is not directly measured but is calculated by dividing 1) the activity in units/ml of the enzyme sample by 2) the concentration of protein in that sample, so the specific activity is expressed as units/mg. The specific activity of a sample of pure, fully active enzyme is a characteristic of that enzyme. The specific activity of a sample of a mixture of proteins is a measure of the relative fraction of protein in that sample that is composed of the active enzyme of interest. DHAD activity can be measured spectrophotometrically in an end point assay using the 2,4-dinitrophenylhydrazine (2,4-DNPH) method as described in Flint, D.H. and M.H. Emptage, J. Biol. Chem. 263:3558-64 (1988). In this assay, the 2,4-DNPH reacts with the keto group of the 2-ketoisovaleric acid product to form a hydrazone, which is detected by its absorbance at 550 nm. The assay buffer contains 50 mM Tris-HC1, 10 mM MgCl₂, pH 8.0 (TM8 buffer). Sufficient 2,3dihydroxyisovaleric acid is added to the assay buffer so that its final concentration in the assay mix is 10 mM. In each assay, an enzyme containing solution and sufficient substrate containing buffer are mixed so that the final volume is 1 ml. The assay mixture is normally incubated at 37°C for 30 minutes.

- **[0253]** The assay is stopped by adding 250 μl of 10% (W/V) trichloroacetic acid. A few minutes later, 500 μl of a saturated solution of 2,4-DNPH in 1 N HCl is added. The mixture is incubated at room temperature for at least 10 min to allow formation of the hydrazone. Next, 1.75 ml of NaOH is added to solubilize the hydrazone and to precipitate unreacted 2,4-DNPH. A few minutes after the NaOH is added, the assay tubes are placed in a sonicator bath for 10 min to degas. The tubes are then centrifuged in a desk top centrifuge at top speed for 2 min to sediment the precipitate.
- [0254] The absorbance of the supernatant is then read at 550 nm within 1 hour. The absorbance of the sample assays minus the control assays are divided by 2600 (determined from an α -ketoisovaleric acid standard curve) to find the units of enzyme activity in the assay. This assay was used in the Examples described herein in which DHAD specific activity was determined.

[0255] Example 8. Purification and Characterization of DHAD from *S. mutans* expressed in *E. coli*.

- **[0256]** DHAD from *S. mutans* was purified and characterized as follows. Six liters of culture of the *E. coli* Turner strain harboring the pET28a plasmid containing the *S. mutans ilvD* gene were grown and induced with IPTG. The *S. mutans* DHAD was purified by breaking the cells with a sonicator in TM8 buffer (*see* Example 7), centrifuging the crude extract to remove cell debris, then loading the supernatant of the crude extract on a Q Sepharose (GE Healthcare) column and eluting the DHAD with an increasing concentration of NaCl in TM8 buffer. The fractions containing DHAD were pooled, brought to 1 M (NH₄)₂SO₄, and loaded onto a Phenyl-Sepharose column (GE Healthcare) equilibrated with 1 M (NH₄)₂SO₄. The DHAD was eluted with a decreasing concentration of (NH₄)₂SO₄. The fractions containing DHAD were pooled, concentrated to ≤ 10 ml, loaded onto a 35 x 600 cm Superdex-200 column (577 ml bed volume) (GE Healthcare) column, and eluted with TM8 buffer. As judged by SDS gels, the purity of the *S. mutans* DHAD eluted from the Superdex column was estimated to be $\geq 90\%$.
- [0257] The UV-visible spectrum of the purified S. mutans DHAD is shown in Figure 3. The number of peaks above 300 nm is typical of proteins with [2Fe-2S] clusters. The S. mutans DHAD was reduced with sodium dithionite, and its EPR spectra was measured at

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various temperatures. Figure 4 shows the EPR spectra measured at temperatures between 20°K and 70°K. The EPR spectrum of the *S. mutans* DHAD is measureable up to 70°K, which indicates that it contains a [2Fe-2S] cluster and not a [4Fe-4S] cluster because the EPR spectra of proteins containing [4Fe-4S] clusters are not observable at temperatures much above 10°K.

[0258] The exact protein content of the batch of purified *S. mutans* DHAD with the highest specific activity using the Bradford protein assay was determined by quantitative amino acid analysis. Combining the activity with the protein content gave a specific activity of 100 units/mg for this batch. The iron content of this batch determined by ICP-MS using methodology known in the art was 2 molecules of iron per molecule of DHAD. This is consistent with this batch of *S. mutans* DHAD containing a full complement of [2Fe-2S] clusters.

[0259] Example 9. Separating the forms of DHAD in yeast crude extract from other proteins in the cell and from each other to measure the amount of DHAD present.

- [0260] DHAD protein in yeast cells exists in the forms of dimers with two Fe-S clusters/dimer, one Fe-S cluster/dimer, and zero Fe-S clusters/dimer. A method to measure the concentration of these three forms of DHAD protein in yeast crude extracts was developed using a Mono Q column and a Source 15 PHE PE 4.6/100 column (both columns obtained from GE Healthcare), and is described below.
- [0261] Frozen yeast cells were thawed, suspended in 50 mM Tris-HC1, 10 mM MgCl₂, pH 8.0 (TM8), then broken by bead beating. The broken cells are centrifuged to remove the cell debris and generate the yeast crude extract.
- [0262] The crude extract was loaded onto a 4 ml Mono Q column attached to an AKTA chromatographic system (GE Healthcare) with the A buffer being TM8 and B buffer being TM8 containing 0.5 M NaCl. The column was equilibrated with A buffer before the sample was loaded. The *S. mutans* DHAD bound to the Mono Q column under these conditions. After the sample was loaded onto the column, the column was washed with 10 mL of TM8 buffer, then the concentration of NaCl in the eluant was increased to 0.22 M NaCl. This was followed by a 30 mL linear gradient from 0.22 M to 0.35 M NaCl. During chromatography, the A₂₁₅ of the column eluate was monitored, and 1 mL fractions

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were collected. The fractions were assayed for DHAD activity. The sum of the activity of the DHAD in the fractions off the Mono Q column was close to that in the crude extract. Good separations using this column were obtained with as much as 5 mL of crude extract representing up to 1 g of yeast cell paste. The DHAD containing fractions were pooled and made 1.35 M in $(NH_4)_2SO_4$ in preparation for chromatography on the PHE column.

- [0263] The Source 15 PHE PE 4.6/100 column was also attached to an AKTA chromatographic system with the A buffer being TM8 containing 1.5 M (NH₄)₂SO₄ and the B buffer being TM8. Before each run the column was equilibrated with 90% A. The pooled fractions from the Mono Q column made 1.35 M in (NH₄)₂SO₄ were loaded onto the PHE column, and at this $(NH_4)_2SO_4$ concentration, the DHAD bound to the column. During chromatography, the A₂₁₅ of the column eluate was monitored, and 1 mL fractions were collected. The DHAD eluted from the column in three peaks when the column was developed with a 30 mL decreasing linear gradient of (NH₄)₂SO₄ from 1.35 M to 0 M. The area of each of the DHAD peaks was determined by integration. This elution scheme was found to be ideal for separating S. mutans DHAD from other yeast proteins that coeluted with it off the Mono Q column. SDS gels run on fractions where the peaks eluted showed that well over 90% of the protein present in these peaks was DHAD when it was expressed at 1% of the soluble protein in yeast cells. The fractions containing each of the three DHAD peaks were pooled separately. Based on the UV-visible absorbent spectrum and the iron and sulfide contents of the DHAD in these peaks, it was determined that the first peak contained DHAD with two [2Fe-2S] clusters/dimers, the second peak contained DHAD with one [2Fe-2S] cluster/dimer, and the third peak contained DHAD with zero [2Fe-2S] clusters/dimers. Thus, in its native state, the S. mutans DHAD enzyme appears to exist as a dimer of two monomeric DHAD proteins.
- [0264] A standard curve relating the amount of DHAD present in a sample to the sum of the area of the three DHAD peaks off the PHE column was obtained as follows. Crude extract from yeast cells containing no *S. mutans* DHAD was spiked with various amounts of purified *S. mutans* DHAD. These extracts were subjected to chromatography on the Mono Q and PHE columns as described above. The area under each of the three DHAD peaks was integrated. The sum of these areas was plotted against the amount of pure DHAD spiked into the yeast crude extracts. The plot was used to derive the following equation:

- [0265] #µg DHAD in sample of crude extract = 0.507 x (summed area counts of the three DHAD peaks)
- **[0266]** The DHAD activity in a crude extract of yeast can be readily determined by the method described in Example 7. The amount of DHAD protein in yeast crude extracts can be determined by the procedure outlined in this Example. With this data, one can calculate the specific activity of the *S. mutans* DHAD protein *per se* in crude extracts according to the procedure in Example 10.

[0267] Example 10. Methods to determine the fraction of DHAD in yeast crude extract loaded with Fe-S clusters.

- [0268] When a purified Fe-S cluster requiring protein contains a full complement of clusters, it will have a characteristic specific activity. As previously mentioned, for S. *mutans* DHAD this specific activity is 100 units/mg when it has a full complement of clusters.
- [0269] A DHAD sample that has on average one Fe-S cluster/per dimer could contain some dimers with two clusters, some dimers with one cluster, and some dimers with no clusters. Alternatively, if cluster addition to a dimer is all or none and on average there is one Fe-S cluster/dimer in a sample, half of the DHAD dimers would have a full complement of clusters and half would be without clusters. From the results in Example 9, we know that all or none behavior is not followed by *S. mutans* DHAD because a species with one cluster per dimer can be isolated. We have found that dimers of *S. mutans* DHAD that have one Fe-S cluster have ½ the activity of dimers with two Fe-S clusters/dimer, *i.e.*, the specific activity of *S. mutans* DHAD with ½ of a full complement of Fe-S clusters is 50 units/mg. This means the absence of an Fe-S cluster in one of the monomers of a dimer does not influence the activity of the other monomer should it contain an Fe-S cluster.
- [0270] With the information obtained with the procedures described in Example 9 and the information described so far in this Example, one can determine the degree of Fe-S cluster loading in a DHAD sample in two different ways.

- [0271] First, one can compare the ratio of the amounts of the three DHAD peaks to determine the relative amount that has two clusters per dimer, one cluster per dimer, and zero clusters per dimer. This gives the degree of cluster loading. For example, if the area of peak 1 off the PHE column was 25%, peak 2 was 50%, and peak 3 was 25% of the sum of the areas of peak 1, peak 2, and peak 3, the percent of the monomers loaded with clusters can be calculated to be 50% according to the following equation:
- [0272] 100* [2 * (area of peak 1) + 1 * (area of peak 2) + 0 * (area of peak 3)]/[2 * (total peak area)] = % DHAD monomers with Fe-S clusters.
- [0273] Second, one can use the specific activity of the DHAD present to calculate the degree of cluster loading. One determines the specific activity by dividing the activity determined as described in Example 7 with the amount of DHAD protein determined as described in Example 9. The specific activity is then divided by 100 U/mg to determine the fraction of monomers loaded with clusters. This fraction is multiplied by 100 to determine the percent DHAD monomers with Fe-S clusters.
- [0274] For example if the specific activity is found to be 50 U/mg, the fraction loaded with clusters is 0.5 and the percent DHAD monomers with Fe-S clusters is 50%.
- [0275] To make such a calculation, the specific activity must be based on the concentration of the DHAD protein in the crude extract (not the total protein). Determining the concentration of *S. mutans* DHAD in the presence of other proteins can be accomplished using methods described in Example 9.

[0276] Example 11. Specific activities and inferred fraction of the DHAD-loaded proteins.

[0277] To determine the fraction of DHAD monomers loaded with Fe-S clusters in several yeast strains grown under different conditions, the methods described above were used. Results are shown in Table 15.

Table 15. Specific Activities and Inferred Fraction of the DHAD Loaded Proteins.

BY Yeast Strain	Growth Conditions	DHAD SA in Crude Extracts (U/mg)	%DHAD is of Crude Extract Protein	% Cluster Occupancy of DHAD
WT	- Ura	0.46	2.3	10
$\Delta FRA2$	- Ura	0.8	2.5	14

$\Delta GRX3$	- Ura	0.99	2.4	23
WT	- Leu	0.82	11	7
$\Delta FRA2$	- Leu	2.2	11	19
$\Delta GRX3$	- Leu	3.5	9.5	31

[0278] These results indicate that under these growth conditions, the level of Fe-S cluster loading in the DHAD in strains lacking FRA2 and GRX3 is higher than in strains containing functional copies of these genes. Thus, a higher fraction of the DHAD protein is in the active form in the deletion strains because the content of Fe-S clusters (which are required for activity) is higher.

[0279] Example 12. Construction of *Saccharomyces cerevisiae* strains PNY1505, PNY1541, and PNY1542.

- [0280] The purpose of this Example was to construct *Saccharomyces cerevisiae* strains PNY1505, PNY1541, and PNY1542. These strains were derived from PNY1503 (BP1064). PNY1503 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversiry Centre, Netherlands). The construction of PNY1503 (BP1064) is described in U.S. Appl. No. 61/368,436, incorporated by reference herein, and in Example 13 below. PNY1505 contains a deletion of the *FRA2* gene. PNY1541 and PNY1542 contain an integration of the *AFT1* gene with the L99A mutation (*AFT1-L99A*) at the *YPRCA15* locus.
- [0281] Deletions/integrations were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and the *URA3* gene for selection of transformants. The *URA3* gene was removed by homologous recombination to create a scarless deletion/integration.
- [0282] The scarless deletion/integration procedure was adapted from Akada *et al.*, *Yeast*, 23(5):399-405 (2006). The PCR cassette for each deletion/integration was made by combining four fragments, A-B-U-C, either by overlapping PCR or by cloning the individual fragments, and gene to be integrated, into a plasmid prior to amplifying the entire cassette by PCR for the deletion/integration procedure. The PCR cassette contained a selectable/counter-selectable marker, *URA3* (Fragment U), consisting of the native CEN.PK 113-7D *URA3* gene, along with the promoter (250 bp upstream of the *URA3* gene) and terminator (150 bp downstream of the *URA3* gene) regions. Fragments

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A (150 bp to 500 bp long) and C (250 bp long) corresponded to the sequence immediately upstream of the target gene (Fragment A) and the 3' sequence of the target gene (Fragment C). Fragments A and C were used for integration of the cassette into the chromosome by homologous recombination. Fragment B (500 bp long) corresponded to the 500 bp immediately downstream of the target gene and was used for excision of the *URA3* marker and Fragment C from the chromosome by homologous recombination, as a direct repeat of the sequence corresponding to Fragment B was created upon integration of the cassette into the chromosome.

[0283] Using the PCR product ABUC cassette, the *URA3* marker was first integrated into and then excised from the chromosome by homologous recombination. The initial integration deleted the gene, excluding the 3' sequence. Upon excision, the 3' region of the gene was also deleted. For integration of genes using this method, the gene to be integrated was included in the cassette between fragments A and B.

[0284] *FRA2* Deletion

The FRA2 deletion (also described in U.S. Appl. No. 61/380,563, incorporated by [0285] reference herein) was designed to delete 250 nucleotides from the 3' end of the coding sequence, leaving the first 113 nucleotides of the FRA2 coding sequence intact. An inframe stop codon was present 7 nucleotides downstream of the deletion. The four fragments for the PCR cassette for the scarless FRA2 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). FRA2 Fragment A was amplified with primer oBP594 (SEQ ID NO: 961) and primer oBP595 (SEQ ID NO: 962), containing a 5' tail with homology to the 5' end of FRA2 Fragment B. FRA2 Fragment B was amplified with primer oBP596 (SEQ ID NO: 963), containing a 5' tail with homology to the 3' end of FRA2 Fragment A, and primer oBP597 (SEQ ID NO: 964), containing a 5' tail with homology to the 5' end of FRA2 Fragment U. FRA2 Fragment U was amplified with primer oBP598 (SEQ ID NO: 965), containing a 5' tail with homology to the 3' end of FRA2 Fragment B, and primer oBP599 (SEQ ID NO: 966), containing a 5' tail with homology to the 5' end of FRA2 Fragment C. FRA2 Fragment C was amplified with primer oBP600 (SEQ ID NO: 967), containing a 5' tail with homology to the 3' end of FRA2 Fragment U, and primer oBP601 (SEQ ID NO: 968). PCR products were purified with a

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PCR Purification kit (Qiagen). FRA2 Fragment AB was created by overlapping PCR by mixing FRA2 Fragment A and FRA2 Fragment B and amplifying with primers oBP594 (SEQ ID NO: 961) and oBP597 (SEQ ID NO: 964). FRA2 Fragment UC was created by overlapping PCR by mixing FRA2 Fragment U and FRA2 Fragment C and amplifying with primers oBP598 (SEQ ID NO: 965) and oBP601 (SEQ ID NO: 968). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The FRA2 ABUC cassette was created by overlapping PCR by mixing FRA2 Fragment UC and amplifying with primers oBP594 (SEQ ID NO: 968). The FRA2 ABUC cassette was created by overlapping PCR by mixing FRA2 Fragment AB and FRA2 Fragment UC and amplifying with primers oBP594 (SEQ ID NO: 961) and oBP601 (SEQ ID NO: 968). The PCR product was purified with a PCR Purification kit (Qiagen).

[0286]

Competent cells of PNY1503 were made and transformed with the FRA2 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research; Orange, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30°C. Transformants with a fra2 knockout were screened for by PCR with primers oBP602 (SEQ ID NO: 969) and oBP603 (SEQ ID NO: 970) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was grown in YPE (yeast extract, peptone, 1% ethanol) and plated on synthetic complete medium supplemented with 1% ethanol and containing 5-fluoroorotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR with primers oBP602 (SEQ ID NO: 969) and oBP603 (SEQ ID NO: 970) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the FRA2 gene from the isolate was demonstrated by a negative PCR result using primers specific for the deleted coding sequence of FRA2, oBP605 (SEQ ID NO: 971) and oBP606 (SEQ ID NO: 972). The correct isolate was selected as strain CEN.PK 113-7D MATa ura3A::loxP his3A pdc6A pdc1A::P[PDC1]-DHAD|ilvD Sm-PDC1t pdc5A::P[PDC5]-ADH|sadB Ax-PDC5t $gpd2\Delta$::loxP fra2 Δ and designated as PNY1505 (BP1135).

Primer Name	SEQ ID NO	Primer Sequence
oBP594	961	agctgtctcgtgttgtgggttt
oBP595	962	cttaataatagaacaatatcatcctttacgggcatcttatagtgtcgtt
oBP596	963	gcgccaacgacactataagatgcccgtaaaggatgatattgttctatta
oBP597	964	tatggaccctgaaaccacagccacattgcaacgacgacaatgccaaacc

Table 16. Primers used in the FRA2 Deletion

oBP598	965	tccttggtttggcattgtcgtcgttgcaatgtggctgtggtttcagggt
oBP599	966	atcctctcgcggagtccctgttcagtaaaggccatgaagctttttcttt
oBP600	967	attggaaagaaaaagcttcatggcctttactgaacagggactccgcgag
oBP601	968	tcataccacaatcttagaccat
oBP602	969	tgttcaaacccctaaccaacc
oBP603	970	tgttcccacaatctattaccta
oBP605	971	tactgaacagggactccgcga
oBP606	972	tcataccacaatcttagacca

[0287] <u>YPRCA15 Deletion and AFT1-L99A Integration</u>

- **[0288]** The YPRCΔ15 locus was deleted and replaced with AFT1-L99A along with the native promoter region (800 bp) and terminator region (800 bp) from AFT1. The scarless cassette for the YPRCΔ15 deletion- AFT1L99A integration was first cloned into plasmid pUC19-URA3MCS (described in U.S. Appl. No. 61/356,379, incorporated by reference herein). The vector is pUC19 based and contains the sequence of the URA3 gene from *S. cerevisiae* CEN.PK 113-7D situated within a multiple cloning site (MCS). pUC19 (American Type Culture Collection, Manassas, VA; ATCC# 37254) contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for URA3, the sequences from upstream (250 bp) and downstream (150 bp) of this gene are present for expression of the URA3 gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.
- [0289] The DNA encompassing the URA3 coding region along with 250 bp upstream and 150 bp downstream of the URA3 coding region from *Saccaromyces cerevisiae* CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Netherlands) genomic DNA was amplified with primers oBP438 (SEQ ID NO: 1033), containing *BamH*I, *AscI*, *PmeI*, and *FseI* restriction sites, and oBP439 (SEQ ID NO: 1034), containing *XbaI*, *PacI*, and *NotI* restriction sites. Genomic DNA was prepared using a Gentra Puregene Yeast/Bact kit (Qiagen). The PCR product and pUC19 were ligated with T4 DNA ligase after digestion with *BamH*I and *XbaI* to create vector pUC19-URA3MCS. The vector was confirmed by PCR and sequencing with primers oBP264 (SEQ ID NO:1031) and oBP265 (SEQ ID NO: 1032).
- [0290] YPRCΔ15 Fragment A was amplified from genomic DNA, prepared as above, with primer oBP622 (SEQ ID NO: 973), containing a KpnI restriction site, and primer oBP623 (SEQ ID NO: 974), containing a 5' tail with homology to the 5' end of

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YPRCA15 Fragment B. YPRCA15 Fragment B was amplified from genomic DNA with primer oBP624 (SEQ ID NO: 975), containing a 5' tail with homology to the 3' end of YPRCΔ15 Fragment A, and primer oBP625 (SEQ ID NO: 976), containing a FseI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). YPRC Δ 15 Fragment A - YPRC Δ 15 Fragment B was created by overlapping PCR by mixing the YPRCA15 Fragment A and YPRCA15 Fragment B PCR products and amplifying with primers oBP622 (SEQ ID NO: 973) and oBP625 (SEQ ID NO: 976). The resulting PCR product was digested with KpnI and FseI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. YPRCA15 Fragment C was amplified from genomic DNA with primer oBP626 (SEQ ID NO: 977), containing a NotI restriction site, and primer oBP627 (SEQ ID NO: 978), containing a PacI restriction site. The YPRCA15 Fragment C PCR product was digested with NotI and PacI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing YPRCA15 Fragments AB. AFT1-L99A, along with the native promoter region (800 bp) and terminator region (800 bp) from AFT1, was amplified using pRS411-AFT1(L99A) (described in Example 4 above) as template with primer oBP744 (SEQ ID NO: 979), containing an AscI restriction site, and primer oBP745 (SEQ ID NO: 980), containing a PmeI restriction site. The PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing YPRCA15 Fragments ABC. The entire integration cassette was amplified from the resulting plasmid with primers oBP622 (SEQ ID NO: 973) and oBP627 (SEQ ID NO: 978).

[0291] Competent cells of PNY1503 were made and transformed with the *YPRCΔ15* deletion/*AFT1-L99A* integration cassette PCR product using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30°C. Transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium supplemented with 1% EtOH and containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the *URA3* marker. The deletion of *YPRCΔ15* and integration of *AFT1L99A* were confirmed by PCR with external primers oBP636 (SEQ ID NO: 981) and oBP637 (SEQ ID NO: 982) and with *AFT1-L99A* specific primer HY840 (SEQ ID NO: 983) and external primer oBP637 (SEQ ID NO: 982) using genomic DNA prepared

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with a Gentra Puregene Yeast/Bact kit (Qiagen) and by colony PCR. Correct independent isolates of CEN.PK 113-7D MATa ura 3Δ ::loxP his 3Δ pdc 6Δ pdc 1Δ ::P[PDC1]-DHAD|ilvD_Sm-PDC1t pdc 5Δ ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd 2Δ ::loxP yprc Δ 15 Δ ::AFT1L99A were designated as strains PNY1541 and PNY1542.

Primer Name	SEQ ID NO	Primer Sequence
oBP622	973	aattggtaccccaaaaggaatattgggtcaga
oBP623	974	ccattgtttaaacggcgcgcggatcctttgcgaaaccctatgctctgt
oBP624	975	gcaaaggatccggcgcgccgtttaaacaatggaaggtcgggatgagcat
oBP625	976	aattggccggcctacgtaacattctgtcaaccaa
oBP626	977	aattgcggccgcttcatatatgacgtaataaaat
oBP627	978	aattttaattaattttttttttttggaatcagtac
oBP744	979	aattggcgcgccagagtacaacgatcaccgcctg
oBP745	980	aattgtttaaacgaacgaaagttacaaaatctag
oBP636	981	catttttttccctctaagaagc
oBP637	982	tttttgcacagttaaactaccc
HY840	983	CCAAAATCAGCCCCACGACGGCCATA

Table 17. Primers used in the YPRCA15 Deletion and AFT1-L99A Integration

[0292] Example 13. Construction of *Saccharomyces cerevisiae* strain BP1064 (PNY1503).

- [0293] The strain BP1064 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Netherlands) and contains deletions of the following genes: URA3, HIS3, PDC1, PDC5, PDC6, and GPD2.
- **[0294]** Deletions, which completely removed the entire coding sequence, were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and either a G418 resistance marker or URA3 gene for selection of transformants. The G418 resistance marker, flanked by loxP sites, was removed using Cre recombinase. The URA3 gene was removed by homologous recombination to create a scarless deletion, or if flanked by loxP sites was removed using Cre recombinase.
- [0295] The scarless deletion procedure was adapted from Akada *et al.* 2006 Yeast v23 p399. In general, the PCR cassette for each scarless deletion was made by combining four fragments, A-B-U-C, by overlapping PCR. The PCR cassette contained a

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selectable/counter-selectable marker, URA3 (Fragment U), consisting of the native CEN.PK 113-7D URA3 gene, along with the promoter (250 bp upstream of the URA3 gene) and terminator (150 bp downstream of the URA3 gene). Fragments A and C, each 500 bp long, corresponded to the 500 bp immediately upstream of the target gene (Fragment A) and the 3' 500 bp of the target gene (Fragment C). Fragments A and C were used for integration of the cassette into the chromosome by homologous recombination. Fragment B (500 bp long) corresponded to the 500 bp immediately downstream of the target gene and was used for excision of the URA3 marker and Fragment C from the chromosome by homologous recombination, as a direct repeat of the sequence corresponding to Fragment B was created upon integration of the cassette into the chromosome. Using the PCR product ABUC cassette, the URA3 marker was first integrated into and then excised from the chromosome by homologous recombination. The initial integration deleted the gene, excluding the 3' 500 bp. Upon excision, the 3' 500 bp region of the gene was also deleted. For integration of genes using this method, the gene to be integrated was included in the PCR cassette between fragments A and B.

[0296]

URA3 Deletion

[0297] To delete the endogenous URA3 coding region, a *ura3::loxP-kanMX-loxP* cassette was PCR-amplified from pLA54 template DNA (SEQ ID NO: 986). pLA54 contains the *K. lactis* TEF1 promoter and kanMX marker, and is flanked by loxP sites to allow recombination with Cre recombinase and removal of the marker. PCR was done using Phusion DNA polymerase and primers BK505 and BK506 (SEQ ID NOs: 987 and 988, respectively). The *URA3* portion of each primer was derived from the 5' region upstream of the *URA3* promoter and 3' region downstream of the coding region such that integration of the *loxP-kanMX-loxP* marker resulted in replacement of the *URA3* coding region. The PCR product was transformed into CEN.PK 113-7D using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on YPD containing G418 (100 μg/ml) at 30 C. Transformants were screened to verify correct integration by PCR using primers LA468 and LA492 (SEQ ID NOs: 989 and 990, respectively) and designated CEN.PK 113-7D Δura3::kanMX.

[0298] <u>HIS3 Deletion</u>

- [0299] The four fragments for the PCR cassette for the scarless HIS3 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). HIS3 Fragment A was amplified with primer oBP452 (SEQ ID NO: 991) and primer oBP453 (SEQ ID NO: 992), containing a 5' tail with homology to the 5' end of HIS3 Fragment B. HIS3 Fragment B was amplified with primer oBP454 (SEQ ID NO: 993), containing a 5' tail with homology to the 3' end of HIS3 Fragment A, and primer oBP455 (SEQ ID NO: 994), containing a 5' tail with homology to the 5' end of HIS3 Fragment U. HIS3 Fragment U was amplified with primer oBP456 (SEO ID NO: 995), containing a 5' tail with homology to the 3' end of HIS3 Fragment B, and primer oBP457 (SEQ ID NO: 996), containing a 5' tail with homology to the 5' end of HIS3 Fragment C. HIS3 Fragment C was amplified with primer oBP458 (SEQ ID NO: 997), containing a 5' tail with homology to the 3' end of HIS3 Fragment U, and primer oBP459 (SEQ ID NO: 998). PCR products were purified with a PCR Purification kit (Qiagen). HIS3 Fragment AB was created by overlapping PCR by mixing HIS3 Fragment A and HIS3 Fragment B and amplifying with primers oBP452 (SEQ ID NO: 991) and oBP455 (SEQ ID NO: 994). HIS3 Fragment UC was created by overlapping PCR by mixing HIS3 Fragment U and HIS3 Fragment C and amplifying with primers oBP456 (SEQ ID NO: 995) and oBP459 (SEQ ID NO: 998). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The HIS3 ABUC cassette was created by overlapping PCR by mixing HIS3 Fragment AB and HIS3 Fragment UC and amplifying with primers oBP452 (SEQ ID NO: 991) and oBP459 (SEQ ID NO: 998). The PCR product was purified with a PCR Purification kit (Qiagen).
- [0300] Competent cells of CEN.PK 113-7D Δura3::kanMX were made and transformed with the HIS3 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research; Orange, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30°C. Transformants with a his3 knockout were screened for by PCR with primers oBP460 (SEQ ID NO: 999) and oBP461 (SEQ ID NO: 1000) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was selected as strain CEN.PK 113-7D Δura3::kanMX Δhis3::URA3.

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- [0301] KanMX Marker Removal from the Δ ura3 Site and URA3 Marker Removal from the Δ his3 Site
- [0302] The KanMX marker was removed by transforming CEN.PK 113-7D Δura3::kanMX Δhis3::URA3 with pRS423::PGAL1-cre (SEQ ID NO: 1011, described in US Provisional Application No. 61/290,639) using a Frozen-EZ Yeast Transformation II kit (Zymo Research) and plating on synthetic complete medium lacking histidine and uracil supplemented with 2% glucose at 30°C. Transformants were grown in YP supplemented with 1% galactose at 30°C for ~6 hours to induce the Cre recombinase and KanMX marker excision and plated onto YPD (2% glucose) plates at 30°C for recovery. An isolate was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in and plated on YPD for removal of the pRS423::P_{GAL1}-cre plasmid. Isolates were checked for loss of the KanMX marker, URA3 marker, and pRS423::P_{GAL1}-cre plasmid by assaying growth on YPD+G418 plates, synthetic complete medium lacking uracil plates, and synthetic complete medium lacking histidine plates. A correct isolate that was sensitive to G418 and auxotrophic for uracil and histidine was selected as strain CEN.PK 113-7D Aura3::loxP Ahis3 and designated as BP857. The deletions and marker removal were confirmed by PCR and sequencing with primers oBP450 (SEQ ID NO: 1001) and oBP451 (SEQ ID NO: 1002) for Aura3 and primers oBP460 (SEQ ID NO: 999) and oBP461 (SEQ ID NO: 1000) for Ahis3 using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen).
- [**0303**] <u>PDC6 Deletion</u>
- [0304] The four fragments for the PCR cassette for the scarless PDC6 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). PDC6 Fragment A was amplified with primer oBP440 (SEQ ID NO: 1003) and primer oBP441 (SEQ ID NO: 1004), containing a 5' tail with homology to the 5' end of PDC6 Fragment B. PDC6 Fragment B was amplified with primer oBP442 (SEQ ID NO: 1005), containing a 5' tail with homology to the 3' end of PDC6 Fragment A, and primer oBP443 (SEQ ID NO: 1006), containing a 5' tail with homology to the 5' end of PDC6 Fragment U. PDC6 Fragment U was amplified with primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer OBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer OBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer OBP444 (SEQ ID NO: 1007), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer OBP444 (SEQ ID NO: 1007).

oBP445 (SEQ ID NO: 1008), containing a 5' tail with homology to the 5' end of PDC6 Fragment C. PDC6 Fragment C was amplified with primer oBP446 (SEQ ID NO: 1009), containing a 5' tail with homology to the 3' end of PDC6 Fragment U, and primer oBP447 (SEQ ID NO: 1010). PCR products were purified with a PCR Purification kit (Qiagen). PDC6 Fragment AB was created by overlapping PCR by mixing PDC6 Fragment B and amplifying with primers oBP440 (SEQ ID NO: 1003) and oBP443 (SEQ ID NO: 1006). PDC6 Fragment UC was created by overlapping PCR by mixing PDC6 Fragment U and PDC6 Fragment C and amplifying with primers oBP444 (SEQ ID NO: 1007) and oBP447 (SEQ ID NO: 1010). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC6 ABUC cassette was created by overlapping PCR by mixing PDC6 Fragment AB and PDC6 Fragment UC and amplifying with primers oBP440 (SEQ ID NO: 1003) and oBP447 (SEQ ID NO: 1007). The PCR product was purified with a PCR Purification kit (Qiagen). The PDC6 ABUC cassette was created by overlapping PCR by mixing PDC6 Fragment AB and PDC6 Fragment UC and amplifying with primers oBP440 (SEQ ID NO: 1003) and oBP447 (SEQ ID NO: 1010). The PCR product was purified with a PCR Purification kit (Qiagen).

- [0305] Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 were made and transformed with the PDC6 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30°C. Transformants with a pdc6 knockout were screened for by PCR with primers oBP448 (SEQ ID NO: 1012) and oBP449 (SEQ ID NO: 1013) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6::URA3.
- [0306] CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6::URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR and sequencing with primers oBP448 (SEQ ID NO: 1012) and oBP449 (SEQ ID NO: 1013) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC6 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC6, oBP554 (SEQ ID NO: 1014) and oBP555 (SEQ ID NO: 1015). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 and designated as BP891.

[0307] <u>PDC1 Deletion ilvDSm Integration</u>

[0308]

<u>I De l'Deletion no Dem integration</u>

The PDC1 gene was deleted and replaced with the *ilvD* coding region from Streptococcus mutans ATCC #700610. The A fragment followed by the *ilvD* coding region from Streptococcus mutans for the PCR cassette for the PDC1 deletion-ilvDSm integration was amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and NYLA83 (described in US Provisional Application No. 61/246709) genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). PDC1 Fragment A-ilvDSm (SEQ ID NO: 1053) was amplified with primer oBP513 (SEQ ID NO: 1016) and primer oBP515 (SEQ ID NO: 1017), containing a 5' tail with homology to the 5' end of PDC1 Fragment B. The B, U, and C fragments for the PCR cassette for the PDC1 deletion-ilvDSm integration were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). PDC1 Fragment B was amplified with primer oBP516 (SEQ ID NO: 1018) containing a 5' tail with homology to the 3' end of PDC1 Fragment A-ilvDSm, and primer oBP517 (SEQ ID NO: 1019), containing a 5' tail with homology to the 5' end of PDC1 Fragment U. PDC1 Fragment U was amplified with primer oBP518 (SEQ ID NO: 1020), containing a 5' tail with homology to the 3' end of PDC1 Fragment B, and primer oBP519 (SEQ ID NO: 1021), containing a 5' tail with homology to the 5' end of PDC1 Fragment C. PDC1 Fragment C was amplified with primer oBP520 (SEQ ID NO: 1022), containing a 5' tail with homology to the 3' end of PDC1 Fragment U, and primer oBP521 (SEQ ID NO: 1023). PCR products were purified with a PCR Purification kit (Qiagen). PDC1 Fragment A-ilvDSm-B was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm and PDC1 Fragment B and amplifying with primers oBP513 (SEQ ID NO: 1016) and oBP517 (SEQ ID NO: 1019). PDC1 Fragment UC was created by overlapping PCR by mixing PDC1 Fragment U and PDC1 Fragment C and amplifying with primers oBP518 (SEQ ID NO: 1020) and oBP521 (SEQ ID NO: 1023). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC1 A-ilvDSm-BUC cassette (SEQ ID NO: 1054) was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm-B and PDC1 Fragment UC and amplifying with primers oBP513 (SEQ ID NO: 1016) and oBP521 (SEQ ID NO: 1023). The PCR product was purified with a PCR Purification kit (Qiagen).

- [0309] Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 were made and transformed with the PDC1 A-ilvDSm-BUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30°C. Transformants with a pdc1 knockout ilvDSm integration were screened for by PCR with primers oBP511 (SEQ ID NO: 1024) and oBP512 (SEQ ID NO: 1025) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC1 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC1, oBP550 (SEQ ID NO: 1026) and oBP551 (SEQ ID NO: 1027). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm-URA3.
- [0310] CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm-URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion of PDC1, integration of ilvDSm, and marker removal were confirmed by PCR and sequencing with primers oBP511 (SEQ ID NO: 1024) and oBP512 (SEQ ID NO: 1025) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm and designated as BP907.

[0311] PDC5 Deletion sadB Integration

- [0312] The PDC5 gene was deleted and replaced with the *sadB* coding region from *Achromobacter xylosoxidans*. A segment of the PCR cassette for the PDC5 deletion-sadB integration was first cloned into plasmid pUC19-URA3MCS.
- [0313] pUC19-URA3MCS is pUC19 based and contains the sequence of the URA3 gene from *Saccaromyces cerevisiae* situated within a multiple cloning site (MCS). pUC19 contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for URA3, the sequences from upstream and downstream of this gene were included for expression of the URA3 gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.
- [0314] The DNA encompassing the URA3 coding region along with 250 bp upstream and 150 bp downstream of the URA3 coding region from *Saccaromyces cerevisiae* CEN.PK

113-7D genomic DNA was amplified with primers oBP438 (SEQ ID NO: 1033), containing BamHI, AscI, PmeI, and FseI restriction sites, and oBP439 (SEQ ID NO: 1034), containing XbaI, PacI, and NotI restriction sites, using Phusion High-Fidelity PCR Master Mix (New England BioLabs). Genomic DNA was prepared using a Gentra Puregene Yeast/Bact kit (Qiagen). The PCR product and pUC19 (SEQ ID NO: 1056) were ligated with T4 DNA ligase after digestion with BamHI and XbaI to create vector pUC19-URA3MCS. The vector was confirmed by PCR and sequencing with primers oBP264 (SEQ ID NO: 1031) and oBP265 (SEQ ID NO: 1032).

[0315]

The coding sequence of sadB and PDC5 Fragment B were cloned into pUC19-URA3MCS to create the sadB-BU portion of the PDC5 A-sadB-BUC PCR cassette. The coding sequence of sadB was amplified using pLH468-sadB (SEQ ID NO: 1051) as template with primer oBP530 (SEQ ID NO: 1035), containing an AscI restriction site, and primer oBP531 (SEQ ID NO: 1036), containing a 5' tail with homology to the 5' end of PDC5 Fragment B. PDC5 Fragment B was amplified with primer oBP532 (SEQ ID NO: 1037), containing a 5' tail with homology to the 3' end of sadB, and primer oBP533 (SEQ ID NO: 1038), containing a PmeI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). sadB-PDC5 Fragment B was created by overlapping PCR by mixing the sadB and PDC5 Fragment B PCR products and amplifying with primers oBP530 (SEQ ID NO: 1035) and oBP533 (SEQ ID NO: 1038). The resulting PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. The resulting plasmid was used as a template for amplification of sadB-Fragment B-Fragment U using primers oBP536 (SEQ ID NO: 1039) and oBP546 (SEQ ID NO: 1040), containing a 5' tail with homology to the 5' end of PDC5 Fragment C. PDC5 Fragment C was amplified with primer oBP547 (SEQ ID NO: 1041) containing a 5' tail with homology to the 3' end of PDC5 sadB-Fragment B-Fragment U, and primer oBP539 (SEQ ID NO: 1042). PCR products were purified with a PCR Purification kit (Qiagen). PDC5 sadB-Fragment B-Fragment U-Fragment C was created by overlapping PCR by mixing PDC5 sadB-Fragment B-Fragment U and PDC5 Fragment C and amplifying with primers oBP536 (SEQ ID NO: 1039) and oBP539 (SEQ ID NO: 1042). The resulting PCR product was purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC5 A-sadB-BUC cassette (SEQ ID NO: 1055) was created by amplifying PDC5 - 107 -

sadB-Fragment B-Fragment U-Fragment C with primers oBP542 (SEQ ID NO: 1043), containing a 5' tail with homology to the 50 nucleotides immediately upstream of the native PDC5 coding sequence, and oBP539 (SEQ ID NO: 1042). The PCR product was purified with a PCR Purification kit (Qiagen).

- [0316] Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm were made and transformed with the PDC5 A-sadB-BUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose) at 30°C. Transformants with a pdc5 knockout sadB integration were screened for by PCR with primers oBP540 (SEQ ID NO: 1044) and oBP541 (SEQ ID NO: 1045) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC5 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC5, oBP552 (SEQ ID NO: 1046) and oBP553 (SEQ ID NO: 1047). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB-URA3.
- [0317] CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB-URA3 was grown overnight in YPE (1% ethanol) and plated on synthetic complete medium supplemented with ethanol (no glucose) and containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion of PDC5, integration of *sadB*, and marker removal were confirmed by PCR with primers oBP540 (SEQ ID NO: 1044) and oBP541 (SEQ ID NO: 1045) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB and designated as BP913.
- [**0318**] <u>GPD2 Deletion</u>
- [0319] To delete the endogenous GPD2 coding region, a gpd2::loxP-URA3-loxP cassette (SEQ ID NO: 1057) was PCR-amplified using loxP-URA3-loxP PCR (SEQ ID NO: 1052) as template DNA. loxP-URA3-loxP contains the URA3 marker from (ATCC # 77107) flanked by loxP recombinase sites. PCR was done using Phusion DNA polymerase and primers LA512 and LA513 (SEQ ID NOs: 1029 and 1030, respectively). The GPD2 portion of each primer was derived from the 5' region upstream of the GPD2 coding region and 3' region downstream of the coding region such that integration of the loxP-URA3-loxP marker resulted in replacement of the GPD2 coding region. The PCR

product was transformed into BP913 and transformants were selected on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose). Transformants were screened to verify correct integration by PCR using primers oBP582 and AA270 (SEQ ID NOs: 1048 and 1049, respectively).

[0320] The URA3 marker was recycled by transformation with pRS423::P_{GAL1}-cre (SEQ ID NO: 1011) and plating on synthetic complete media lacking histidine supplemented with 1% ethanol at 30°C. Transformants were streaked on synthetic complete medium supplemented with 1% ethanol and containing 5-fluoro-orotic acid (0.1%) and incubated at 30 C to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in YPE (1% ethanol) for removal of the pRS423::P_{GAL1}-cre plasmid. The deletion and marker removal were confirmed by PCR with primers oBP582 (SEQ ID NO: 1048) and oBP591 (SEQ ID NO: 1050). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB Δgpd2::loxP and designated as BP1064.

[0321] Example 14. Shake flask experiment to measure 2,3-dihydroxyisovalerate accumulation and isobutanol production.

- [0322] The purpose of this Example was to show the effect on accumulation of the isobutanol pathway intermediate 2,3-dihydroxyisovalerate (DHIV) and show isobutanol production in isobutanologen strains with an integrated copy of the *AFT1-L99A* gene or a *FRA2* deletion compared to the parent strain. Strains were transformed with isobutanol pathway plasmids pYZ090 (SEQ ID NO: 984; described in U.S. Appl. No. 61/368,436, incorporated by reference herein) and pLH468 (SEQ ID NO: 985; described in U.S. Application No. 61/246,844, incorporated by reference herein). These plasmids are also described briefly, as follows.
- [0323] pYZ090 (SEQ ID NO: 984) was constructed to contain a chimeric gene having the coding region of the alsS gene from *Bacillus subtilis* (nt position 457-2172) expressed from the yeast CUP1 promoter (nt 2-449) and followed by the CYC1 terminator (nt 2181-2430) for expression of ALS, and a chimeric gene having the coding region of the ilvC gene from *Lactococcus lactis* (nt 3634-4656) expressed from the yeast ILV5 promoter

(2433-3626) and followed by the ILV5 terminator (nt 4682-5304) for expression of KARI.

- [0324] pLH468 (SEQ ID NO: 985) was constructed to contain: a chimeric gene having the coding region of the ilvD gene from *Streptococcus mutans* (nt position 3313-4849) expressed from the *S. cerevisiae* FBA1 promoter (nt 2109 3105) followed by the FBA1 terminator (nt 4858 5857) for expression of DHAD; a chimeric gene having the coding region of codon optimized horse liver alcohol dehydrogenase (nt 6286-7413) expressed from the *S. cerevisiae* GPM1 promoter (nt 7425-8181) followed by the ADH1 terminator (nt 5962-6277) for expression of ADH; and a chimeric gene having the coding region of the codon-optimized kivD gene from *Lactococcus lactis* (nt 9249-10895) expressed from the TDH3 promoter (nt 10896-11918) followed by the TDH3 terminator (nt 8237-9235) for expression of KivD.
- [0325] A transformant of PNY1503 (parent strain) was designated PNY1504. A transformant of PNY1505 (*fra2* deletion strain) was designated PNY1506. Transformants of PNY1541 and PNY1542 (*AFT1-L99A* integration strains) were designated PNY1543 and PNY1544, for PNY1541, and PNY1545 and PNY1546, for PNY1542.
- [0326] Strains were grown in synthetic medium (Yeast Nitrogen Base without Amino Acids (Sigma-Aldrich, St. Louis, MO) and Yeast Synthetic Drop-Out Media Supplement without uracil and histidine (Clontech, Mountain View, CA)) supplemented with 100mM MES pH5.5, 0.2% glucose, and 0.2% ethanol. Overnight cultures were grown in 15 mL of medium in 125 mL vented Erlenmeyer flasks at 30°C, 225 RPM in a New Brunswick Scientific I24 shaker. 18 ml of medium in 125 mL tightly-capped Erlenmeyer flasks was inoculated with overnight culture to an OD₆₀₀ 0.5 and grown for six hours at 30°C, 225 RPM in a New Brunswick Scientific I24 shaker. After six hours, glucose was added to 2.5%, yeast extract was added to 5 g/L, and peptone was added to 10 g/L (time 0 hours). After 24 and 48 hours, culture supernatants (collected using Spin-X centrifuge tube filter units, Costar Cat. No. 8169) were analyzed by HPLC (method described in U.S. Patent Appl. Pub. No. US 2007/0092957, incorporated by reference herein) and LC/MS. Glucose and isobutanol concentrations were determined by HPLC. DHIV was separated and quantified by LC/MS on a Waters (Milford, MA) AcquityTQD system, using an Atlantis T3 (part #186003539) column. The column was maintained at 30°C and the flow

rate was 0.5 mL/min. The A mobile phase was 0.1% formic acid in water, and the B mobile phase was 0.1% formic acid in acetonitrile. Each run consisted of 1 min at 99% A, a linear gradient over 1 min to 25%B, followed by 1 min at 99%A. The column effluent was monitored for peaks at m/z=133 (negative ESI), with cone voltage 32.5V, by Waters ACQ_TQD (s/n QBA688) mass spectometry detector. DHIV typically emerged at 1.2 min. Baseline separation was obtained and peak areas for DHIV were converted to μ M DHIV concentrations by reference to analyses of standards solutions made from a 1 M aqueous stock.

[0327]

7] Table 18 shows the DHIV molar yield (moles of DHIV per moles of glucose consumed) and isobutanol titer of the *AFT1-L99A* strains (PNY1543, PNY1544, PNY1545, and PNY1546) and the *FRA2* deletion strain (PNY1506) compared to the parent strain background (PNY1504) at 24 and 48 hours. *AFT1-L99A* expression or the *FRA2* deletion both led to approximately a 50% decrease in the accumulation of DHIV.

Strain	<u>24 Hr</u>	<u>48 Hr</u>	<u>24</u>	<u>48 Hr</u>
	DHIV Yield	<u>DHIV Yield</u>	<u>Hr</u>	<u>Isobutanol</u>
	(mol/mol)	<u>(mol/mol)</u>	<u>Isobutanol</u>	<u>Titer (g/L)</u>
			<u>Titer (g/L)</u>	
PNY1504	0.044	0.035	3.7	4.2
PNY1543-	0.017	0.015	4.1	5.8
PNY1544				
PNY1545-	0.019	0.018	4.6	5.5
PNY1546				
PNY1506	0.022	0.020	3.8	4.7

Table 18. DHIV molar yield and isobutanol titer.

Data are the average of two independent flasks, for PNY1504 and PNY1506, and two independent transformants for the *AFT1-L99A* strains (PNY1543-PNY1544 and PNY1545-PNY1546).

[0328] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

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7.16 3.05.0 2.16.5 3.05.0 2.16.5 3.05.0 1.17 3.05.0 1.07.14	·710 ·2703 ·3050 ·2465 ·2697 ·2691 ·177 ·2999 ·2034 ·720 2773 ·3520 ·3552 ·3148 ·3779 ·2691 ·4004 ·3668 ·720 2755 ·3342 ·3748 ·3779 ·2617 ·3064 ·3668 ·720 2755 ·3344 ·45 ·96 ·359 ·117 ·3699 ·2644 ·720 2756 ·182 ·1874 ·1869 ·2644 ·2644 ·720 2756 ·182 ·1874 ·1669 ·2644 ·2644 ·720 2756 ·184 ·187 ·1874 ·3529 ·117 ·3699 ·2244 ·720 2756 ·184 ·1874 ·1366 ·1274 ·3699 ·2244 ·720 2756 ·1847 ·1666 ·1679 ·1677 ·3699 ·2246 ·720 2756 ·177 ·369 ·177 ·3699 ·234 ·720 </td <td>7.76 3.7103 3.050 2.463 2.697 2.553 1.674 1.0968 653 6133</td>	7.76 3.7103 3.050 2.463 2.697 2.553 1.674 1.0968 653 6133
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44(V)	45(G) -	<u>46(l)</u>	(V)74	48(N)	49(M) -	50(W)	51(W) -	52(D)	53(1)	54(1)	55(P)

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3631 275	4286 275	-2271 275	1443 275	-40 Pcs 275	1826 275	-12G 275	-3771	-1718 275	-36 275	-263 275	-2403 275
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- <u>3859</u> -466	-3936 -466	16 -166	-3178 -466	2824 -466	-2197	-2025 -466	2080 -465	-3237 -466	-442	-203 +66	-601 -466
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Table 12

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112	-859	-865	-454 117	-507	-2938	-701	-1476	117	-876	-1759	-1555
358	-662 359	-754 359	411 359	-1117 359	-2593 359	1138 359	-2680 359	-3260	-801 359	-3311 358	-1361 359
98	-2646 96	-351 96	756 96	-1484 96	-3232 96	7 <u>957</u> 96	-3113 96	-3716 96	-2451 96	-3835 96	2826 96
4 <u>5</u>	-2310 45	28.46	33 45	-1230 45	-2039 45	-2010	-2864 45	-3456 45	-2218 45	-3608 45	-2475
394	-2(132 394	-1796	-1690 394	-21.02 394	-3014 394	-1909 394	-3362 394	-3774 394	-2208 394	-3638 394	2418 394
275	-1721 275	-201	1435 275	-1634 275	-1551 275	-1557 275	-3112 275	-3626 275	- 1906 275	-3689 275	-2024 275
-720	-2407 -720	-1486 -720	-456 -720	3130 -720	-3956	-2187 -720	-87	-319 -720	-1202 -720	-462 -720	-2984 -720
-2604	-3257 -466	-2331	-1207 -136	-156 466	-4365 -466	-3074 -466	-209 -466	588 -486	-1646 -466	-508 -168	-3741 -468
210	-2637 210	68 210	210 210	-1513 210	-2621 210	-2302 210	-3208 210	-3712 210	-2445 210	-3783 210	-2730 210
-2815 -825 +	-2972] -626	-2400 -526 *	-1037 -626 *	195 -626 *	-626	-2842 -626 *	2274 -626 *	2287 -626	-575 -628	-520	-3611 -626
-1378			-234 106 -1378*	+ 8781- 106 1378-	-2197 106 -1378	-2139 106 -1378*	-2577 106 -1378*	-3433 106 -1378*	-2106 106 -1378	-3655 106 -1378*	-2534 106
2229 399 -701				-2041 399 -701	-2466 399 701	3399 -701	-3487 399 -701	-3988 399 -701	-1577 399 -701	-3984 389 -701	900 900
-2833 -381 -1115	-3250 -381 -1115			-341 -381 -1115	-4140 -381 -115	-3160 -381	1821 -381 -1115	-1511 -381 -1115	-2116 -381	-1668 -381 -1115	-3654 -381
-62 43 -894				-1828 43 -804		-2298 43 -891	-3459 43 -594	-3373 43 -894	-2665 43 -894	-3936 43 -894	2375 43
-8150		-48 233 8150	-733 -733 -8150	-2417 233 -8150		-2334 233 -8150	-3950 233 -8150	-4292 233 -8150	-2608 233 -8150	-4298 233 -6150	-2169 233
-2211 -500 -7106				-469 -500 -7103	- 34 32 -500 -7108	-930 -500 -7105	-1144 -500 -7108	-1294 -500 -7108	-500 -7109	-1323 -500 -7108	-1755 -500
-987 -149 -16				410 -149 -16	-2764 -149 -16	2142 -149 -16	-1514 -149 -16	-1743 -149 -16	-149	-1764 -149 -16	-1157 -149i
116(C)	117(G)	- -	119(W)	120(M)	121(D)	122(G)	123(V)	124(V)	125(A)	126(1)	127(G)

122

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Table 12

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3668 -4222	-294	3464 -3718			-3710 -3552			-3001 -2968			-3253 -2909			-2513 -2207				-204 -249		-3068 -4222	-294 -249		-3256 -2983	-294 -249		-2002 -1868	-294248			-294 -249		-3412 -3228	-294 -249
4004		3158			-4046			-3331			-3392	- 1			-369			-369			-369		-1849	-369		1156				-369			-3691
	211		117		-2938				117		-2439				117		-3279			-2981			-659	117		-1726	117		-2146	117		-857	117
-2339	353	-2793	359		-2593				359		-2224			-2911	359		-3182	359		-2839	359		2146	359		-2843	358		-3164	358		-664	350
-3779	36	-3332]	96		-3232	98		-465	96		-2608			-2996	96		-3895	36		-9779	96		-2302	96		-3088	96		-3521	96		-2648	96
3748	¢3	-4030	45		-2039	45		-1260	10 10		-2205	45		-3046	45		-3781	45		-3748	45		-1906	45		-2717	45		-3445	45		.2327	LC V
-3352	394	-3363	394		-3014	394		-3039	394		26.92-	394		-3425	394		BACK	296		-3955	394		18981 -	394		-3401	394		-3674	394		-2034	9.06
-3520	2759	-3631	275		-1551	275		-2043	275		3026	275		-3293	275		STONE.	775			275		-1516	275		-325/6	275		-3562	275		-1739	1320
-4212	-726	-3569	-720		-3956	-720		-2339	-720		-9437	-726		2043	-720		4165	0.02		6124	-720		-2134	-720		8645	-720		693	-720		-2381	000
4671	466	-3859	-466		-4365	465		3387	-456		-3936	466		-596	466		44904	A50		1674	-486		-3021	-466		1235	-466		-659	-166		-3226	001
3953	210	-4167	210		-2621	210		9754	210		-2365	210		-3121	210		0220	010		2052	210		-2174	210		-3293	210		-3581	210		-2650	
-476:	-828	-3513	-626	*	4505	-528	*	-3603	-626	*	-3943	-626	*	-1047		*	12024		*	1784	16	-	-2796	-626	2	1553	-628	-	3564	-626		-2930	000
	:06 -1378	-3545	106	-1378	76:0-	106	-1378'	-1570	106	-1378	-2267	106	-1378 *	-2824	106	-1378	0460	10404	-1378	0070	100:	-1378	-2058	106	-1378	-2608	:06	-13/8	-3266	1:06	-1378	2380	
374.8	399 -701	-2763	399	-701	-2466	390	101-	1070-	3991	-701	-2364	399	-701	-3105	399	-701	12005	000	-701	Jaco de Como	1995 1999	101-	1008	399	101-	3668	396	102-	-3632	309	-701	1051	200
-4328	-1115	-3712	-381	91.13-	-3140	-381	-1115	1743	-381	-:115	•	-381	1 1	-1525	-381	-115	44041		-1115	Sector.		-115		-381	-1115	-669		91.11-	-1676	-381		2222-	ļ
-3623	43 -894	-43581	43	-394	12004	£.4	-894	204.44	43	-894	1748	43	-894	-3594		-894			168-	10000		Ŷ	1901-0-		ų	-35704		-894	-3840		-894	-2572	
3304	233 -8150	-41021	233	-8150			ιφ	1900		-8150		233				-8150		1	-8150		PUCC-			238		-4142		Ĩ	-3971		Ŷ	2445	
			8		CEVE			7061				-500				-7108		- 1	-7108		1080Z-	'		-500			500			-500			
-2594	-149	-2476	-149	-16	1/02/01	-149	-16	2620	140	-16	11210	-149	-16	1 -2408	149	-16		1567-	91-		1011	-16	100000000000	1451-	-16	1700	-149	-16	11602- 1	-149	-16	1 3403	
128(C)		130(C)	12/2-1		VU/VE F	17/001		1.71.200	1.1.2	_	4.3076.0	-		1 3 C/MAY	1.00		1.00	134(1)			13((1))		14/961	1200-		1.97/MA	-		138013	-		130/01	NJAC 1

123

WO 2011/103300

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	103300		, 1	124		, , ,			T/US20	r
146	147 148	149	150	151	152	153	154	155	156	157
-249	-3228 -249 -2186 -249	-1410	-27(1) -249	-1396 -249	-4064 -249	-249	-2163 -249	2460 -249	-2326 -249	4713 -249
-294	-3412 -294 -2523 -264	- 1599 -294	-3633	-1771 -294	-3594	-3279 -284	-2365	28	-2700	-294
-286 -366	-1981 -369 -369 -369 -369	-151 -369	-3692	1960 -369	-4087 -369	-369	-369	-1245	-369 -369	-3071
117	-857 117 -1505	952 117	-1757	-1024	-3279	-684	-1841 117	-2120	-1118	-3527
3439	-664 359 -1520 358	-2153 359	-1371 359	-1795 359	-3182 359	359	-3092	-2535 359	-1464 369	-3418 359
-3414 96	-2648 96 3394	-2375 96	-2071	1701 96	-3895 96	-2313 96	-3317	-2702 96	-2766	3341 96
-2910 45	-2327 45 -324 45	-2065	-724 45	-1638 45	-3781	-1915 45	-3014 45	-2372 45	-2559 45	-3101 45
-3711 394	-2034 394 -2357 394	-2922 394	-2182 394	-2692 394	394 394	-1901 394	-36 1 3 394	-3309 394	-2640 394	-3772 394
-3855	-1733 275 1858 275	2574 275	275	-2067 275	-3491 275	-1515 275	-3490	-2502 275	-2349	3027 275
3828	-720 -720 -1916 -720	2 2 56	-2870	-92	-720	-2159 -720	-720	3167 -720	-720	-2526 -720
1864 -466	-3226 -466 -2625 -2625	2225 -466	-3531	455	-4490 -466	-3048 -466	246 -466	-746 -466	-1122 -166	-2516 -466
210	-2650 210 373 210	-2473 210	210	-1668 210	-3859 210	-2165 210	-3425 210	-3082	-2771 210	3638 210
1242 -629	-2330 -626 + -2795	* -626 *	-3622 -626	2235 -626 +	-4621 -626	-2326 -626 -	* -626 -	-1077 -626	417 -626	-3000 -626
-3013 106 -1378	-2380 106 -1378* -708	-1378* -1923 106 -1378*	-1040 106 -1378 *	-1601 106 1378 -	-3468 106 -1378*	-2068 106 -1378*	-2991 106 -1378*	-628 106 -1378*	-2460 106 -1378*	-1112 106 -1378
-4222 399	1051 396 -701 -701 -2171	-701 -3(124 -701	-1643 399 -701	-2675 399	-2925 399 -701	1011 329	-3841 399	-3385 399 -701	-2150 399 -701	-3652 399
-498 -381 -115	-3222 -381 -1115 -3069	-1115 -474 -381 -115	-3621 -381 -1115	-1041 -381 -115	-41E1 -381	-3082 -381	-963 -381 -115	-381	-1633 -381 -1115	239 -381
-4012 43 -894	-2572 43 -894 -953	-894 -894 -2819 -894	78 43 -894	-2239 43 -894	-3706 43 -894	-2192 43 -894	-3728 43 -894	-3473 43 -894	-2994 43 -894	-3919 43 -894
-4598 233 -8150	-2445 -233 -8150 -1399	-815C -3386 -8150	2055 233 -8150	-2825 233 -8150	-3420 233 -8150	-2267 233 -8150	-4195 233 -8150	-3724 233 -8150	-3200 233 -8150	-3701 233 8150
-1891 -500 -7108	-1036 -500 -7108 -7108 -7108	-7108 -1256 -500 -7108	-3306 -500 -7108	-921 -500 -7108	-2878 -500 -7108	-940 -500 -7108	-1492 -500 -7108	-1797 -500 -7108	-1028 -500 -7108	-2868 -500 -7108
-2325 -149 -16	3183 -149 -1588	-16 -1448 -149 -16	-1662 -149 -16	-1066 -149 -16	-2931 -149 -16	1568 -149 -16	-1680 -149 -16	-2204 -149 -16	1265 -149 -16	-3462 -149 -16
140(M)	141(A) - - 142(H)		144(N) -	145(!)	146(P) - -	147(S) -	148(1)	149(F)	150(V) -	151(Y)

SUBSTITUTE SHEET (RULE 26)

Table 12

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0 2011/	103300				125				r t i	.7US201	. 1/0232
158	159	160	161	162	163	164	165	166	167	168	169
-249	-4222 -249	-2850	-2493	-1446 -249	-1763 -249	-4222	-1670 -249	3456 -249	-1476	-4222	-1881 -249
-3868 -294	-3668 -294	-3152 -294	-294	-2073	-2369	-3668 -294	-2335 -294	42051 -294	-2161 -294	-3668 -294	-294
-309	-4004 -369	-1760 -369	369	-1517	-1657 -369	-4004 -369	-1623 -369	-1799	-1602 369	-4004 -369	-2093
2981 117	-2981	117	117	-459	-805	-2981	-687	-2577	-457	-2981	-1014
-2839	-2839 359	1110 359	-3194 359	359	855 359	-2839	-588 359	-2788	-364	-2839 359	-1013 359
3779 96	-3779	-2247 96	-3521 96	96 96	-941 96	-3779 96	-233	-2960	-211 96	-3779 96	1334 96
-3748 45	-3748 45	-1969	-3445	362 45	-560 45	-3748 45	150 45	-2514 45	1288	-3748	108 45
3352 394	-3352 394	-1958 394	-3674 394	-1566 394	2341 394	-3352 394	-1689 384	-3534 394	- 1536 394	-3352 394	- 1989 394
3320	-3320 275	-1561 275	-3562	-120	-689 275	-3320	2250	-2551 2755	941 275	-3320	-556 275
-4212 -720	-4212	-2064 -720	-720	-1022 -720	-720	-42.12	-1306 -720	-1217 -720	-1082 -720	-4212 -720	-1482 -720
-4671 -466	4671	-2875 -466	-659 -456	-1918 -466	-2233	-4671 -466	-2183 -466	1047	-1991 -466	-4671 -466	-2295 466
-3953 210	-3953 210	-2170 210	3561 210	1769 210	-586 210	-3953 210	1178 210	-3306 210	210	-3953 210	2262
-4761 -326 -	-4761 -520	-2560 -626 *	-626 *	-1974 -626	-2122 -620	-4761 -626	-2252 -626	-1691 -626	-2043 -626	-4761 -626 +	-2459 -626
-3452 :06 -1378	-3462 106 -1378*	-2074 106 -1378	-3289 106 -1378*	1278 106 -1378*	2143 106 -1378	-3462 106 -1378	2466 106 -1378*	-469 106 -1378*	-114 -1378 *	-3462 106 -1378*	-326 106 -1378
3767 399 -701	3747 3995 - 701	-1242 399 -701	-3532 399 -701	-1477 399 -701	-1463 399 -701	3742 399 701	-1512 339 -701	-3611 399 -701	1403 399 -701	3747 399 -701	-1912 399 -701
-1115	-4328 381 1115	-2900 -381 -1115	-1676 -381 -1115	-2260 -381	-2204 -381	-4323 -381	-2513 -381	941 -381 -1115	-2295	-1328 -381	-2856 -381 -1115
-3523 43 -894	-3623 43 -894	-2229 43 -894	-894 -894	1010 43 -894	-557 43 -894	-3823 43 -894	1118 43 -894	-3748 43 -894	279 43 -894	-3623 43 -894	2043 43 -894
-3304 233 -8150	-3304 233 -8150	-2225 233 -8150	-3971 233 -8150	-384 233 -8160	-711 233 -8150	-3304 233 -8150	-114 233 -8150	-3850 233 -8150	779 233 -6150	-3304 233 -8150	912 233 8150
-2690 -500 -7106	-2630 -500 -7109	-976 -530 -7108	-1746 -530 -7108	-1924 -500 -7103	-1502 -500 -7196	-2690 -500 -7103	-2193 -500 -7108	-2139 -500 -7106	-1981 -500 -7198	-2690 -500 -7108	-2365 -500 -7108
-2594 -149 -16	-2594 -149 -16	-359 -149 -16	-2091 -149 -16	861 -149 -56	-655 -149 16	-2594 -149 -16	-744 -149 -36	-2672 -149 -16	386 -149 -16	-2594 -149 -16	-1144 -149 -16
152(G)	153(G)	54(1)	155(I) - -	156(H) -	157(P) -	158(G) -	159(H)	160(W)	161(K)	162(G) -	163(K)

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SUBSTITUTE SHEET (RULE 26)

Table 12

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				126	j				17US201	
170	171	173	174	175	176	177	178	179	180	181
-2105 -249	-2224 -249 -2090	-2417 -249	-2677 -249	-3025 -249	-2055 -249	349 -249	-3385 -249	-3224 -249	-2345	-4222 -249
-2880 -294	-2247 -294 -2853 -2853	-2961	-3074	-294	-2390	-739 -294	-3563	-3412 -294	-2609	-3668 -294
-369	-36 -369 -369 -2184 260	1570 -369	369	-2413	147 4 -369	-2566	-3722	-1980 369	-3692	-4004
117	-2296 117 2122	-1761	-1917	-1316	-641	-3420	-2750	- 850	-1/31	-2961
740 359	-3647 -359 -860 -860	-3249 359	-2628	359	1075 359	-3490 359	-2479	-656 358	-3143 359	-2339 359
-1006	-3586 96 -1124		-3437 96	-2703 96	-2005 96	-3645 96	-2560 96	-2637	-3518 96	-3779 96
-206 45	-3098 45 -255	-3283 45	-3363 45	-2483 45	-1674 45	-3350 45	-1842 45	-2309	-3214 45	-3748 45
-1885 394	-3847 394	-3730 394	-33999 394	-2360 394	-1946 394	-3780 394	-2959] 394	-2(128 394	-3667 394	-3352 394
-178 275	-4023 275 2171	-75 -3633 -275	-3246	-1973 275	-1517	-32.89	-1531] 2755	-1727 275	-3537 275	-3320
-1877 -720	558 -720 -1825	-120	-1076 -720	-720	-1137	-1900	-720	-2377 -720	-720	-4212 -720
-2606 -466	2050 466 -2640	-400) 1259 -455	-1154 -466	-3497 -466	-1832 -466	-1801 -466	-466 -466	-466	1486 -466	-4671 -466
1084 210	-3865 210 -521	210 210 210	210	-2686 210	-1399	4038 210	-2128 210	-2633 210	-3589 210	-3953 210
-2750 -326	1569 -626 +	-529 -626	403 -626	-3294 -628	-1264 -626	-2315 -626 -	-4105 -626 -	-2934 -626	1633 -626	-4761 626
-1378	-3250 106 -1378 * -635	106 -1378 * -3274 106 -1378 *	-3231 106 -1378*	-2474 106 -1376 *	-1704 106 -1378*	-1431 105 -1378*	-2043 106 -1378	-2368 106 -1378*	-3162 106 -1378*	-3462 106 -1378 '
-1527 399 -701	-4366 396 -701 -1499	-701 -701 -701 -701		-1640 399	-1344 309 -701	-701	-2458 399 -701	1544 399 -701	-3901 399 -701	37.67 399 -701
-2957 -381 -115	-617 -381 -1115 -2870	-381 -115 -1210 -1115	-2037 -381 -1115	-3185 -361 -115	-1993 -381	-1115 -1115	-3966 -381 -1115	-3222 -381	-1216 -381	.4328 -381
174 43 -894	-4155 -43 -894 133	43 -894 -3573 -894	-3689 43 -894	-2543 43 894	-2082 43 -894	-4232 43 -894	-894	-2551 43 -894	-3793 43 -894	3623 43 -894
233 233 -8150	4674 233 -8150 1806	233 -8150 -8150 -8150	-3750 233 -8150	-2333 233 -8150	-2371 233 -8150	-4020 233 -8150	-896 233 -8150	-2429 233 -8150	4255 233 -8150	-3304 233 -8150
-2610 -530 -7108	-1922 -500 -7109 -2427	-530 -7138 -1390 -500 -500		-1462 -500 -7108	-824 -500 -7103	-2776 -500 -7108	-3508 -500 -7108	-1031 -500 -7108	-1342 -500 -7108	-2690 -500 -7108
-1091 -149 -16	-2387 -149 -16 -1021	-149 -16 -1830 -1830 -16		-897 -149 16	2440 -149 -16	-3342 -149 -16	-2641 -149 -16	29843 - 149 - 16	-1769 -149 -16	-2594 -149 -16
164(D)	165(L) - - 166(N)		168(V)	169(S) -	170(A)	171(F) -	172(E) - -	173(A)	74(V)	175(G) -

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SUBSTITUTE SHEET (RULE 26)

	176(G)		(M)/2/1		,	11/071									101/101		ĒE			<u>.</u>	E	20		4 04/67	-		106/07		1	1 BELDA	101001	-	107/11	(-1)/01
C10 C10 <td>671-</td> <td>-16</td> <td>1 -1652</td> <td>- 149</td> <td>-16</td> <td>165 1</td> <td>140</td> <td>-16</td> <td>10011</td> <td>1921-1</td> <td>-16</td> <td>1 42421</td> <td></td> <td>-16</td> <td>504</td> <td>-149</td> <td>-16</td> <td>Loon 1</td> <td>-1024</td> <td>-16</td> <td>1020</td> <td>-149</td> <td>-1.6</td> <td>1 245</td> <td>-149</td> <td>-16</td> <td>1001</td> <td>1971-</td> <td>-16</td> <td>12041</td> <td>-149</td> <td>-16</td> <td>12672</td> <td>121 -</td>	671-	-16	1 -1652	- 149	-16	165 1	140	-16	10011	1921-1	-16	1 42421		-16	504	-149	-16	Loon 1	-1024	-16	1020	-149	-1.6	1 245	-149	-16	1001	1971-	-16	12041	-149	-16	12672	121 -
(168 2510 (158) (150) (-2116	-7108	-1707	-500	-7108	-752	-200	-7108	1000	12801-	-7108	10510	5003	-7103	-2610	-200	-7108	1674	-200	-7105	175.01	-500	-7108	147/16-	-500	-7108	0000	-500	ľ		-500	-7108	0.75	
QIN Sign TON ZINN ZINN <thzinn< th=""> <thzinn< th=""> ZIN</thzinn<></thzinn<>	-413	-8150	-2340	233	-8150	-1251	233	-6150	100	400-	-8150	1206	666	-8150	1246	233	-8150	102.11	233	-8150	1224	233	-8150	095	233	-8150	020	233	-6150	0673	233	-8150	3750	2520-
358 113 218 <td>1096</td> <td>-994</td> <td>-1879</td> <td>43</td> <td>-894</td> <td>102-</td> <td>13</td> <td>-894</td> <td>1-1-</td> <td><u>6</u></td> <td>68- 498-</td> <td>1460</td> <td>43</td> <td>-804</td> <td>10801</td> <td>43</td> <td>-894</td> <td>107.22</td> <td></td> <td>168-</td> <td>1+4+</td> <td>43</td> <td>-894</td> <td>1000</td> <td>43</td> <td>-894</td> <td>17485</td> <td>E.</td> <td>-894</td> <td>11010</td> <td>÷++</td> <td>-894</td> <td>STATE</td> <td>20.79</td>	1096	-994	-1879	43	-894	102-	13	-894	1-1-	<u>6</u>	68 - 498-	1460	43	-804	10801	43	-894	107.22		168-	1+4+	43	-894	1000	43	-894	17485	E.	-894	11010	÷++	-894	STATE	20.79
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1958 1588 1969 177 100<	3991	-701	-2733	395	102-	-1670	399	:02-	3 V O V V	1000	IOZ-		22	101-		1		9702	300	-701	10061	399	101-											
-2189 1969 -2004 -710 2.13 1964 -177 1525 1790 177 1525 2190 2004 2191 2004 <t< td=""><td>1599</td><td>-1378</td><td>2013</td><td>106</td><td>-1378</td><td>-535</td><td>106</td><td>-1378</td><td>100 State 100 State</td><td>2</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>1</td><td>11</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></t<>	1599	-1378	2013	106	-1378	-535	106	-1378	100 State 100 State	2												1	11											
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-572 -17.70 -22.93 -24.9 -1577 -13.25 2539 -29.4 -24.9 -1577 -13.25 2594 -24.9 - -177 -36.9 -29.4 -24.9 - 117 -36.9 -29.4 -24.9 - 117 -36.9 -29.4 -24.9 - 117 -36.9 -29.4 -24.9 - -1764 -271.3 -36.9 -29.4 -24.9 -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - -117 -36.9 -29.4 -24.9 - <td></td> <td></td> <td>-</td> <td></td> <td>l</td> <td></td> <td>ł</td> <td></td> <td></td> <td></td> <td></td>			-												l															ł				
-11/101 -24/13 -1010 -5009 -2204 -249 -369 -294 -249			1-									-						1									1							
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-249 -249 -249 -249 -249 -249 -249 -249	-284		258	7.94		-1236	-204									-294																		
183 185 185 185 186 189 189 189 189 189 189 189 189	-249		2136	-249		-812	-249		-1340	-249		-2234	-249		1494	-249		-2059	-249		-1961	-249		-1556	-249					'	-249			
	2		183			184			185			186			187			188			189			190			191			192			193	

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134	195 196	197	198	199	200	201	202	203	204	205
-249	-20()5 -249 -2409	-249 -:1769 -:249	-1774 -249	-1431 -249	-3320	-1967	-4064 -249	-3226 -249	-1522 -249	4222 249
-1900	-2710 -294 -2703	-294 -2325 -284	-2142 -294	-2307 -294	-3405 -294	-294	-3594	-3477 -294	-2070	-3668 -294
-1309	-2000 -369 2833	-369 -426 -369	-1817 -369	-2522	-2183	379	-4087	-2130 -369	-1243 -369	-4004 -369
111	-923 117	-1157	-1038	-1546 117	-1387	-1375	117	-905 117	1019	-2981
359	-763 359 359	358 -1340 359	-1123 359	-1352 359	-1216 359	-1809 359	-3182 359	1217 359	450	-2839 359
6/- 96	-3568	96 -1238 96	22 13 96	-1085 96	-2944 96	-2830 96	-3695 96	-2583	-585 96	.3779 96
21 <u>7</u>	-233 45 -3280	45 -797 45	1553 45	-721	-2798 45	-2710 45	-3781 45	-2177 45	1404 45	-3748
0000 0000	-1820 394 -3693	394 -2295 394	-2070	-2259 394	-2447 394	-2696) 394	394	-2042	1285 394	-3352 394
275	1123 275 -3551	275 -922 275	-747 275	3635 275	-2236 275	-2078 275	-3491 275	-1599 275	-434 275	-3320 275
-720	-1655 -720 -140	-720 -897 -720	-1272 -720	-720	-2662 -720	-720	-4165 -720	-720	-935 -720	-4212
-486	-2462 -466 1168	-1273 -1273 -466	-2037	-2845 -466	-3257 -466	-706	-4490 -466	-466	-1759 -466	-1671 465
210	-442 210 -3614	210 210 210	210	-735 210	-3028 210	-2922 210	-3859 210	-2506 210	-159 210	-3953 210
-1650 -626	-2467 -628 *	-626 - 1892 -626 *	-2093 -626	-2925 -628 +	-2840 -626	1565 -626 -	-626 -626	-3172 -626 *	-1629 -626	-4761 -626
1735 106 -1378*	-615 -615 -1378* -1378*	106 -1378* -1068 1068 -1378*	-358 106 -1378*	2032 106 -1378	-2735 106 -1378	-2432 106 -1378	-3468 106 -1378 *	-2272 106 -1378	-477 106 -1378*	-3462 106
-1467 399 -701	-701 -3919 -701 -3916	<u>-701</u> -701 -701 -701	-203P 399 -701	1837 399 701	-1726 399 -701	-2525 339 -701	-2925 399	395 101-	102- 1995 19851-	37 <i>6</i> 3
- 1925 - 1115 - 1115	-1115 -381 -1115 -1311	-1115 -1115 -381 -381	-25441 -381 -115	-2011 -381 -115	-3267 -321 -1115	-1440 -381 -1115	-4181 -381	-3315 -361 -1115	-1969 -381 -1115	-4328 -381
1031 43 -894		-894 -894 -894	-620 43 -894	-403 43 894	-3040 43 -894	-3314 43 -894	-3706 43 -894	-2180 43 -894	-249 43 -894	-3623 43
-409 -8150		233 -8150 -734 -8150	-1398 233 -8150	-261 233 -8150	-2846 233 -8150	-3609 233 -8150	-3420 233 -8150	-1983 233 -8150	-705 233 -8150	330 4 233
-1713 -500 -7108		-7108 -7108 -7108 -7108	3528 -500 -7108	-2527 -500 -7158	-1472 -500 -7103	-7108	-2878 -500 -7108	-1115 -500 -7108	-1347 -500 -7108	-2690 -500
-479 -149 -16		-149 -149 -149 -16	-1182 -149 -16	-1478 -149 -16	2438 -149 -16	-1220 -149 -16	-2931 -149 -16	-477 -149 -16	1653 -149 -16	-149
138(F)	189(G)		192(C)	(N)E01	194(A)	195(C) -	196(P)	- - -	198(A)	99(G)

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206	207	208	209	211	213	214	215	216	217
-2990	-3718 -249	-4222 -2:49	-249 -249 -2207 -249	4423 -249 -3156	-249 -3320 -249	-2909 -249	-3156 -249	-1925 -249	-3049 -249
-3265 -294	-294	-264	-3668 -284 -2513 -294	336 -294	-294 -3405 -294	-3253 -294	-3286	-2039	-3306 -294
-1857	-3158 -369	-4004 -369	-4004 -369 -1398 -369	-2669 -369	-369 -2183 -369	-3392	-2197 -369	-474 -369	-1850 -369
-660	-2860	-2981	-2981 117 -2552 117	-3474 117	117] -1387 117]	-2439	117	-2274	117
2235 359	-2793 359	-2639	-2339 359 -2911	-1463	359 -1216 359	-2224 359	-1463 358	-3327 359	2652 359
-2300 96	-3332 96	-3779 96	-2996	-3431 96 -2753	95 2944 96	-2603	-2753 96	-3171	-2436 96
-1901 45	-4036	-3748	-3746 45 -3046	-2845 45 -2708	45 -2798 45	-2205 45	-2708 45	-2806 45	-2105
-1598 394	-3363 394	-3352 394	-3352 394 394 394 394	-3921 394	394 -2447 384	-2932 394	-2800 394	-3671 394	-1936 394
-1511 275	-3631 275	-3320	-3320 275 -3294	-2744 2755 -2311	275 -2236 -275	4205 276	-2311 275	-3680	1613 275
-2144 -720	-3569 -720	-1212 -720	-4212 -720 -720	-1973 -720	-720 -2662 -720	-3437 -720	-720	-720	-2205 -720
-466	-3859 -466	-4671 -466		-1928 -456 -3105	-466 -3257 -466	-3936 -466	-31(35 -466	1451 1466	-3065 466
2168	-4167 210	-3953 210	-3953 210 -3121 210	-3963 210 210	210 -3023 210	-2365 210	-2788 210	-3385	-2384 210
-2810 -326	-3513 -626	-4761 -626	-4761 -626 * -1047 -626	-2517 -626 - -2693	-626 * -5340 -626	-3943 -626	-2693 -626	-626 *	-2781 -626
-2056 106 -1378	-3545 106 -1378*	-3462 106 -1378 *	-3462 106 -1378* -2824 106	-1378 - 404 106 -1378 1378	106 -1378* -2736 106 -1378*	-2267 106 -1378*	-2659 106 -1378*	-2868 106 -1378*	-2205 106 -1378
1488 399 -701	-2765 395 - 701	102- 636 8768	3743 399 -701 -31015 -31015	-701 -4028 -399 -701	399 -701 -1726 -701	-2364 399 -701	-1622 396 -701	-4051 399 -701	-1197 399 -701
-3068 -381	-3712 -381 -1115	-4328 -381	-4328 -381 -1115 -1115 -1115 -381	-1115 -381 -381 -3163	-381 -1115 -3287 -381 -1115	-3334 -381 -1115	-3163 -361 -1115	-504 -381	-3075 -381 -115
-2183 43 -894	-4358 43 -894	-3623 43 -894	-3623 43 -894 -3594 43	-894 -4379 -4379 -894 -2908	43 -891 -3040 -594	-1748 43 -894	-2906 43 -894	-3834 43 -894	-:2415 43 -894
-2270 233 -8150	-4102 233 -8150	-3304 233 -8150	-3304 233 -8150 -3638 233	-8150 -4146 -8150 -8150	233 -8150 -2846 -2846 -8150	-1458 233 -8150	-2755 233 -8150	4343 233 -8150	-2407 233 -8150
-938 -500 -7106	-5/00	-2690 -500 -7108	-2690 -520 -7108 -2296 -500	-7103 -2700 -500 -7108 -1674	-500 -7108 -1472 -500 -7108	-2655 -500 -7108	-1674 -500 -7108	-1986 -500 -7102	-939 -500 -7108
1870 -149 -16	-2476 -149 -16	-2594 -149 -16	-2596 -149 -16 -2406 -2406	-16 -3590 -149 -16	-149 -16 -16 -149 -16	-2171 -149 -16	-1213 -149 -16]	-2355 -149 -16	2150 -149 16
200(S) -	201(C)	202(G) -	203(G) - - 204(M)	- 205(Y)	- 207(A)	208(N) -	209(1)	210(M)	21!(S) -

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Table 12

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218	219 220	221	223 224	225 226	227 228 229
-2882 -249	-3078 -249 -692 -249	-3365 -249 -1866 -249	-2145 -249 -229 -249	-1874 -249 -3025 -249	-2182 -249 -1942 -249 -249
-3187 -294	-3322 -294 -1445 -294	-3563 -264 -2200	-2111 -294 -3668 -294	-2026 -294 -3310 -294	-2134 -294 -294 -294 -294
-1804	-1842 -369 -369 -369	-3722 -369 -369	-591 -369 -4004	-457 -369 -2413 -369	
1775	-739 117 -1877	2750 117 117 117	-2457 117 -2981 117	-2249 117 -1316 117	-2500 117 117 117 117 117
359	1225 359 -2894 359	-2479 359 -406 359	-3704 359 -2839 359	-3249 359 359	-3793 559 -1769 -1136 -1136 359
-2222 96	-2512 98 -3179 96	-2027	-3489 96 -3779 96	-3097 96 -2703	-3541 -3541 -2130 -2130 -2130 2130
-1903 45	-2205 45 -2821 45	-1842 45 45	-2978 45 -3748	-2769 45 -2483 45	-3016 45 -2082 45 -906
-1941 394	-1948 394 -3492 594	-2950 394 394 394	-3839 394 -3352	-3630 394 -2360 394	-38.80 39.4 39.4 39.4 39.4
-1528	-1658 275 -3197 275	-1531 275 -1640 -1640	-4075 275 -3320 275	-3099 275 -1973 275	-4157 275 -2173 -2173 -2173 -275
-2100	-720 -720 -720	-35555 -720 -813 -720	3 854 -720 -720	-720 -720 -720	2482 -720 -720 -720 -720
-2934 -466	-3071 466 150 -466	-4016 -466 -1398 -486	2513 466 467	1292 -466 -3497 -466	88228 -466 -466 -19:4
2116	-2501 210 -3367 210 210	-2128 -210 -1942 -1942	-3811 210 -3953 -3953	-3259 210 -2656	-3378 210 210 -2060 210 -1059 210
-2851	-2765 -626 - 3030 -526 -	-4105 -626 -499	999 -626 -476: -526	95 -626 -6294	96 -1321 -1321 -526 -526
-2042 106 -1378*	-2274 106 -1378 -2081 106	-1310 -2043 106 -1378* -1698	-3165 106 -1378 -3462 -3462 -1378	-2800 105 -1378 * -2474 106	-3231 -3231 -1378* -1378* -1378* -1378* -767 -767
-1227 399 -701	-1200 -1200 -101 -101 -101 -100	1 1 1 1 1 1 1 1 1 1	-701	-3966 -399 -701 -1640 -399	-4506 -4506 -701 -701 -701 -701 -703 -399
-2959 -381 -111 5	-3075 -381 -1115 -1115 -381 -381	-1115 -3866 -1115 -1115 -1115	-461 -381 -47328 -47328 -4115	-518 -381 -115 -115 -3185 -3185 -1115	-462 -361 -1115 -1710 -381 -1115 -1115 -381
-2162 43 -894	-2533 -2533 -3658 -3658		-4165 43 -3623 43 -894	-3765 43 -594 -543 -2543 -394	-4228 43 -894 -2371 -894 -836 -836
-2190 233 -8150	-2460 -233 -8150 -4067 -233		-4725 -4725 -8150 -3304 -3304 -8150	-4255 233 -4156 -8156 -2333 -8150	-4786 233 -8150 -2473 -2473 -2473 -333 -8150 -865 -865
-979 -500 -7106	-932 -500 -7108 -1546 -500		-2118 -500 -7108 -7108 -7108	-1968 -500 -7108 -7108 -1462 -7108	-2159 -2159 -7109 -7778 -500 -7108 -1670 -500
-344 -149 -16	5648 - 149 - 16 - 1924 - 149		-2576 -149 -16 -16 -149 -149 -16	-2313 -149 -16 -16 -16 -16	-2631] -149] -1501] -1501] -149] -166] -166]
212(S) - -	213(A) - - - - - - -	215(E) - 216(A)	217(M) - - 218(G) -	initial initial initial <	221(L)

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Table 12

	\square					ΠΙ	ΠΙ	\square 1			Π
230	231	232	233	234	235	236	237	238	230	240	241
-3025	-3092 -249	-2833	-890	-2998 -249	·2630 -249	-995	-2552 -249	-1597 -249	-3385 -249	-2986	-1375 -249
-3310	-3349	-3143	-1540	-3243 -294	-3064	-1467	-3334 -294	-2268 -294	-3563	-3001 -294	-1986 -294
-2413	-1884 -369	-1773 -369	-1604 -369	-1893	-1871 -369	-369	-369	-1649	-3722 -369	-3331 -369	376 -369
-1316 117	-749 117	2492	-680	-895	-888	-459	-1366	-597	-2750	-2536	117
2462 359	359 359	358 358	-776 359	-722	-715 359	-670 359	2377 359	-514 359	-2479 358	-2604 359	-409 353
-2703 96	-2467 96	-2184 96	-158 96	-2391	-1921 96	-856 96	-1824 96	418	-2560 96	-465 96	1555 96
-2463	-2145 45	-1866 45	-183	-2093 45	-1468 45	-200	-730	2600 45	-1842 45	-1260	343
-2360 394	-1948 394	-1936 394	-1652 394	3041 394	-1995 394	689 394.	-2068 394	-1637 394	-2059 394	-3039 394	-1560 394
-1973 275	-1621 275	-1510 275	1904 275	-1663 275	1555 275	-577	-428	-125 275	-1531 275	-2048 275	.135 275
-2780	-2265 -720	-2048 -720	38.99 720	-2210	-2054 -720	-206 -720	-2436 -720	-1189 -720	3555	-2839 -720	
-3497 -466	-3122 -466	-2878 -466	-1364 -466	-2986 -466	-2843 -466	250 466	-3186 -486	-2080	-4016 -466	-3357 -468	-1735 -466
-2586	-2426 210	-2067 210	-132 210	210	-1661 210	-283 210	-1165 210	210	-2128 210	3784 210	1452 210
-3294 -626	-2935 -626 *	-2594 -626 *	-1209 -626 *	-2683 -626	-2580 -626	-485 -526	-5176 -626	-2067 -626	-4165 -628	-526 *	-1786 -626
-2474 106 -1378 *	-2237 106 -1378*	-2003 106 -1378*	2436 106 -1378*	-2188 106 -1378*	-1673 106 -1378	-441 106 -1378 *	-1057 106 -1378-	-267 106 -1378*	-2043 106 -1378*	-1570 106 -1378*	-95 106 -1378 *
-1640 399 -701	-1204 399	-1229 389 -701	-1695 399 -701	-1346 399 -701	-1345 399 -701	-1669 399 -701	-1536 339 -701	177 399 -701	2458 399 - 701	-2791 399 701	-1474 399 -701
-3185 -381 -1115	-3120 -381 -115	-2912 -381	-1228 -381 -1115	-3037 -381 -115	-2868 -381	-1116 -381	-3286 -381	-2337 -381 -1115	-366 -361 -1115	-3743 -381 -1115	-2095 -381 -115
-2543 43	-2422 43 -394	-2124 43 -894	-343 43 -894	-2293 43 -894	-1377 43 -894	43 43 -891	-117 43 -894	971 43 -864	43 43 494	-2046 43 894	983 43 -894
-2333 233 -8150	-2367 233 -8150	-2176 233 -8150	-710 233 -8150	-2241 233 -8150	-1299 233 -8150	-834 233 -8150	2333 233 -8150	-100 233 -8150	-£96 233 -8150	-2461 233 -8150	-415 233 -8150
-1462 -500 -7106	-954 -500 -7108	-975 -500 -7108	-1440 -520 -7108	-1100 -500 -7103	-1235 -500 -7108	-1046 -500 -7108	-2412 -500 -7108	-1983 -500 -7108	-3308 -500 -7198	-2961 -500 -7108	-1802 -500 -7108
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224(S) -	225(S) -	226(S)	227(M)	228(P)	229(A)	230(E)	231(D)	232(Q) -	233(E) -	234(K)	235(R) -

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Table 12

- 236(U)	-	237(E)	-	-	238(C)	-		239(E) C	منعط		11 240(E)		,	H2 241(S)			242(G)		, Л	E 243(F)	26	,	244(V)	1	-	245(I)	,	-	246(V)		-	247(E)	
-149 -149	L	-1225	- 149	-16	1375	-149	-16	635	-149	-16	1693	-149	9:-	1884	-149	-16	2267	-149	-16	-876	-149	-:6	2339	-149	-16	-1827	-149	-16	-1178	-149	-16	-508	-140
-1505 -500 -7106	lone	-2866	-500	-7108	3262	-500	-7108	-1790	-500	-7108	-2044	-500	-7108	-635	-500	-7108	-1043	-500	-7108	-2037	-500	-7108	-967	-500	-7108	-1398	-500	-7108	-1448	-500	-7108	-1976	-500
233	he o-	1694	233	-8150	-2620	233	-8150	1055	233	-8150	-252	233	-8150	-1962	233	-8150	-2338	233	-8150	-829	233	-8150	-2970	233	-8150	-4307	233	-8150	-1943	233	-8150	840	626
443 43	heo-	134.6	43	-894	-2108	43	-894	376.1	43	-894	2548	43	-394	-1576	43	-894	-2528	43	-894	1490	43	-594	-2766	43	-894	-3331	43	-894	-1452	43	-894	1547	EP
- 38		-3149	-381	-1115	-827	-381	-1115	-2018	-381	-115	-2437	-381	-115	-1634	-381	-115	-3253	-381	-1115	-2474	-381	-1115	-1878	-381	-1115	-1099	-381	-1115	-1776	-381	-1115	-2280	381
399		-1532	366	10/-	-1866	66E	i:02-	-1464	399	-701	-1542	399	-701	-1436	399	-701	2622	399	-701	-1766	339	102-	-1847	399	1:01-	0836-	3969	102-	-2261	309	102-	-1393	000
1378-	6101-	-671	106	-1378	-1267			-263	106	-1378*	-329	106	-1378	-1320	106	-1378+	-2375	106	-1378	-229	106	-1378	-2252	106	-1378	-3142	:06	-1378	-1140	106	-1378*	117	505
-978	-	-2975	-626	*	1631	-526	Ť	1191	-626	3	-2133	-626	*	1041	-628	1	-2975	-626		-2109	-626	8	32	-626	-	12260	-628	*	-227	-626	Ť-	-2029	A2A
210	_	-630			-1811	210		28	210		151	210		-1409	210		-2839	1 1		1209	210		-2541	210		-3619	210		1966	210	Η	14:00	010
466		-2902	466		-599	-466		-1767	-466		-2120	-466		-1453	-466		3260	466		44	-466		-1209	-466		1835	-466		-1260	466		-1934	ARE
-720		-2101	-720		-10	-720		946	-720		-1274	-726		-781	-720		-2410	-720		-1198	-720		-018	-720		69	-720		-816	-720		-1077	1004
275		-150	275		-1674	275		641-	275		-244	275		-1293	275		-1725	275		-124	275		12087	275		-3579	275		-1444	275		1158	575
996 19		-1935	394		-2137	394		-1637	39.4		-1738	394		-1922	394		-2035	394		-1829	394		-2399	394		-3671	394		-2448	394		-1531	505
2 <u>6</u> 4		-293	45		-1531	45		135	1 <u>7</u>		89	45		-1241	45		-2311	4		205	45		-2316	45		-3177	45		-902	45		330	125
-15U2		-1299	98		-1786	96		-481	96		946	96		-1606	96		-2848	96		2226	96		-2545	96		-3511	96		-540	96		-253	QR
359		1884	359		-1034	359		-520	359		-646	359		6266	359		-663	359		-775	353		-1157	359		-3178	359		-1496	358		-378	350
111		-1241	117		790	117		-553	117		212-	117		-597	117		-860	117]		-768	117		-971	117		-1731	117		-1176	117		-454	117
969 969		-2496	-369		249	-369		-1300	696.		4671-			699-	-369		-2005	-369		-1753	-369		2345	-369		1918	369		2692	-369		262	280
-294		-3093	-294		-1361]	-294		-2077	-294		-2305	-294		-2036	-294		-3458	-294		-2143	-294		-2605	-294		-2524	294		-2161	-294		-2163	-2GA
-249		.2248	-249		-1010	-2-49		1445-	-249		-1686	-249		-1656	-249		-3250	-249		-1047	-249		-2251	-249		.23:0	-249		-1764	-249		-1471	070
797		243			244			245			246			247			248			249			250			251			252			253	
	4	. 1									e !												1		1	1							,

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0 2011/	/103300			133		PC1/US201	
254	255	256	257 258	259 260	261 262	263 264	265
-1177 -249	-2258 -249	-2140	-1803 -249 -2320 -249	-2273 -249 -1804 -249	-4004 -249 -2158 -249	-2317 -249 -2295 -249	-2091 -249
-1442 -294	-2397	-2692 -294	-2159 -204 -3161 -294	-2449 -294 -2168 -294	-3594 -294 -2546	-3161 -294 -2549	-2084
-369	1383 -369	-2368	-1603 -369 -2564 -369	546 -369 -1580 -369	-4087 -369 -1998 -369	-2552 -369 -369 -369	-172
-1052	-1839 117	-1256	-1263 -117 -1315 -1375	-2021 117 -1443 117	-3279 117 -1053 117	-13:6 117 -1959 117	-2149
-1685	-3250 359	-1144 359	-1423 359 -1050 359	-3413 359 -1618 359	-3182 359 258 359	553 359 -359 -359	.3 378 359
-2010 96	-3494 96	-177 96	287 96 -1379 96	-3557 96 96	-3695 96 261A	-1301 96 -3555	3406 96
-1715 45	-3109 45	-175	-282 45 2106 45	-3187 45 45	-3781 45 -543	1342 45 -3239 45	-2916 45
-2591	-3687 394	-2066	-2303 394 -1879 394	-3785 394 -2436 394	38분1 394 -2102 394	-1079 394 -3783 394	-3685 394
-2:04 275	-3656 275	-464	-1075 275 -190	-3779 275 -1231	-3491 275 -1042 275	-19.0 2755 -3718 -3718	3808 275
-720	257 -720	-1854 -720	2576 -720 -720 -720	244 -720 -720 -720	-4105 -720 -723 -720	-2194 -720 97 -720	3.67
1544 -466	2186 466	-2633 -466	-1652 -466 -2977 -2977	1451 456 938 456	-4490 -466 -2489 -466	-2971 -466 -466 -466	1993 466
-2031	-3656 210	2306 210	210 210 210 210	-3706 210 8:334 210	-3859 210 -44 210	-728 -728 -3654 -210	-3709
168 -629 -	-526 +	-2759 -526	-1709 -626 - -3047 -626	3164 -628 + -1603 -626 +	-4621 -626 * -2490 -2490	-3034 -626 -526 -526	2068 -626
-1550 106 -1378]*	-3118 106 -1378*	-576 106 -1378 *	-641 -1378 -737 -737 -737 -737 -737 -737 -737 -	-3236 1978 - -750 -750 -1378	-3468 105 -1378* -996 106 -1378*	-743 106 -1378 -1378 -1378	-1378*
-2567 399 -701	-4013 309 -701	-1811 399 -701	-2216 -701 -701 3399 -701	-4130 399 -701 -2363 359 -701	-2925 339 -701 399	-1547 399 -701 -701 -701	-4164 399 -701
-528 -381	-916 -381 -115	-3071 -381 -115	-2215 -381 -1115 -3210 -381	-91115 381 381 381 381 381	-4181 -381 -1115 -1115 -1115	.3154 361 1115 1115 381 381	-530 -381 -115
-2342 43 -894	-3885 43 -894	2191 43 -894	-1614 -894 -894 -176 -894	-3975 -3975 894 -1191 -1191 -894	-3706 -3706 -394 -894 -394	175 43 -894 -3927 -3927 -394	-3091 43
-2901 233 -8150	-4335 233 -8150	-272 233 -6150	-1711 233 -8150 -8150 -8150	-4434 233 -8150 -1827 -1827 -8150	-3420 233 -8150 -1507 -8150	8164 233 -8150 -4355 -8150	-4572 233 -8150
-991 -500 -7106	-1516 -500 -7108	-2647 -500 -7108	-2059 -500 -7108 -2688 -500 -500	-1632 -500 -7108 -2144 -500 -500	-2678 -500 -7108 -1705 -7105 -7106	-2865 -500 -7108 -1562 -500 -7108	-1821 -500 -7108
1703 -149 -16	-1947 -149 -16	-1322 -149 -16	-1395 -149 -16 -16 -149 -16	-2073 -149 -1670 -1570 -149 -16	-29311 -149 -161 -928 -928 -1491 -161	-1280 -149 -16 -16 -16 -16 -16 -16	-2252 -149 -16
248(M)	249(1)	250(E) -	25:(K) 252(U)	() () () () () () () () () () () () () ((a) (a) (b) (c) (c) (c) (c) (c) (c) (c) (c) (c) (c	257(D) - - - - - -	259(M) -

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Table 12

0 2011	/103300			134		PC1/U	S2011/0252
266	267	263	270	272	274	275	277
-249	-2384 -249	-2138 -249 -249 -249	-158 -249 -3385 -249	-2700 -249 -3320 -249	-2806 -249	-2705 -249 -2524	-2113
-3265 -294	-2484 -294	-26555 -294 -3305 -3305	-1038 -294 -3563	-3633 -294 -3405 -294	-3240 -294	-2999 -294 -2856	-2516 -2516 -294
-2197	-2748 -369	-2369 -369 -1640 -369	-83 -369 -3722	-3092 -369 -369 -369	2276 -369	-1635 -369 -369 -369	-209 369
38.9	-1828	-1271 117 -730	-2012 117 -2750	-1757 117 -1387 117	-1752	117	1344
-1463 359	-2020 359	-1178 358 1831 359	-2876 359 -2479 359	-1371 359 -1216 359	-3359 359	902 959 -3232	-2563 359
-2753	3482 96	-61 96 2463 96	-3127 96 -2560	-2071 95 -2344	-3896 96	-2163 96 -3673	
-2705 45	-173 45	-161 45 -2147 45	-2739 45 -1842 45	-724 45 -2798 45	-3673 45	-1809 45 45 -3403	-2870 45
-2600	-2603 394	-2090 394 -1940 394	-34.R6 -394 -2959 -2959	-2182 394 -2447 394	-3869 394	-1806 394 -3749	-33255 -33255
276	-1353 275	-514 276 -1633 -1633	-3095 275 -1531	275 275 275 275	-3722 275	-1504 275 -3606	-19
-2612 -720	1985 -720	-1837 -720 -720	67 -720 -3555 -720	-2670 -720 -720 -720	-515	-7846 -720 -261	-120 -720
-466	-26455 -466	-2612 -466 -3056 -456	135 -436 -4016 -456	-3531 -466 -3257 -466	-579 -466	-2678 -466 -456 -456	-616 -616
-2789 210	1735 210	26900 210 210 210 210	-3359 210 -2128 -2128	-1272 210 -3028 -3028	-3828 210	-2037 210 -3688 240	-3080
-2598	-2 <u>976</u> -626	-2750 -626 -626 * * -526 *	2063 -626 + -626 + +	-526 -526 -526 -526 -526	- -626 -626	-2392 -628 - 2358 -	-uzu 2276 -626
-2659 106 -1378	-587 106 -1378*	-565 -565 -1378 -2233 -2233	-1706 106 -1378 - -2043 106	-1378 -1046 -1378 -1378 -1378 -1378 -1378	-3726 106 -1378 *	-1940 1940 -1378 -3370	-1378* -1378* -2696 106
-1922 399 -701	-2636 399 - 701	-1857 -701 -701 -701 -701	-3644 399 -701 -2458 399	-701 -1643 -1643 -701 -701 -1726 -1726	-4042 399 -701	-1206 399 -3967 -3967	-701 -701 -3356 -701
-3163 -381 -1115	-3618 -381 -1115	-3083 -381 -1115 -1115 -381 -381	-381 -381 -1115 -3966	-11130 -3621 -3811 -1115 -3287 -381	-1735 -381 -1115	-2747 -351 -1115 -1446 -1446	-1115 -1464 -381
-2908 43 -894	-1460 43 -894	2083 43 -894 -894 -894 -894	-36/7] 43 -804 43 43		-3962 43 -894	-2158 -33 -894 -3858 -3358	-894 -3305 -894
-2755 233 -8150	-2704 233 -8150	-381 -381 -3150 -3150 -3150 -313 -3150 -8150	-4037 233 -8150 -896	-6150 2055 233 -8150 -8150 -2840 -8150	-4325 233 -8150	-2334 -233 -8150 -4286 233	-8150 -3766 -333 -8150
-1674 -500 -7106	2786 -500 -7108	-2635 -500 -7108 -7108 -932 -500	-1686 -500 -7108 -3308 -500	-/108 -3306 -500 -500 -500 -500 -500	-1307 -500 -7108		-7108 -7108 -500 -7108
-1213 -149 -16	-2131 -149 -16	-1349 -149 -16 -16 -16 -149 -16	-2063 -149 -16 -16 -2641	-1662 -1662 -149 -16 -16 -149 -16	-1760 -149 -149	1428 -149 -16 -1745	-166 -1404 -149
260(1) -	261(R)	262(K) - - 263(A)	264(F) - 265(E)	- 	268(I)	269(T) - - - 270(V)	- 271(V)

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Table 12

	1/103300	, 		135			T/US2011/025
278	279	280 281 281	282	283 284	285 286	287	288 289
-1803	-2279 -249	-1426 -249 -4222 -249	-249	-30255 -249 -3156 -249	-2909 -249 -3093	-248	-2182 -249 -1681 -249
-2134	-2626	-1724 -294 -3665 -3665		-3310 -294 -3286 -294	-3263 -294 -3333	-2588	-2154 -294 -294 -294
-369	1990 -369	1777 -369 -4604 -369	-4004	-2413 -369 -369 -369	-3392 -369 -1844	-305 -3699	-608 -369 -3695 -3695
-1053	-929 117	985 117 -2961	-2981	-1316 117 117 117	-2439 117 -747 117	1504 117	-2509 117 117 117
-1220	-1053 359	-1944 358 -2339 359	-2839 359	359 359 359 359	-2224 359 374 874	-1601 359	-3793 359 3591 359
-2078	-2494 96	-2362 96 -3779 96	96 8278-	-2703 96 -2753 95	-2603 96 -2537	*0 -2661 96	-3541 96 -2620
-1928	-2260 45	-2106 45 -3746	-3748 45	-2483 45 45 45	-2205 45 -2240	-2480 45	-3016 45 -2833 45
-2387	-2320 394	-2816 394 -3352 394	-3352 394	-2360 394 -2600 394	-2932 394 -1954	994 394	-3680 394 -3482 394
-1950	-20(i8	-2418 -275 -3320 -3320	-3320	-1973 275 275 -2311 -275	4205 275 -1672	2378 -2378	-4157 275 -2886
-720	-1001 720	198 -720 -720 -720	-4212	-2700 -720 -720	-3437 -720 -720	-720	2482 -720 -720 -720
-466	-1406 -466	2001 466 -1671 -1671	4671	-3497 -466 -3105 -3105	-3936 -465 -3082	001 988 1466	3828 466 -466 -466
-2061	-2487 210	-2418 210 -3953 210	-3953	-2696 210 210 210 210	-2365 210 -2540	-2643 -2643	-3878 210 210 210 210
-487 -626	-165 -628	590 590 -526 + 761 +	-626	-3294 -626 -526 * -526 *	-3843 -626 -626 -626	-020 + -623 + -623	96 -626 -626
1833 1378 -1378	-2211 106 -1378*	-1888 106 -1378 * -3462	-1378* -3462 106 -1378*	-2474 106 -1376 * -2659 106 -1378 *	-2267 -1378 -1378 -2300	-1378* -1378* -2404 -1378*	-3231 106 -1378 * 106 106
-1920 399 -701	-1740 395 -701	-2795 -701 -701		-1640 -701 -701 -1922 -701	-2304 -2304 -701 -701	-701 -701 -701 -701	-4506 -399 -701 -3046 -3999
-1322 -381 -1115	-1943 -381 -1115	-796 -381 -115 -1328 -381	-1115 -4128 -381	-3185 -381 -381 -381 -381 -1115	-3334 -3334 -1115 -1115 -3383	-1115	-462 -381 -1115 -2110 -381
-2412 43 -894		-2733 43 -894 -3623 43	-894 -3623 -3623 -894	-2543 43 -894 -2906 43 -891	-1748 -594 -567	-394 -2908 -894	-4228 -43 -894 -33 -890
-2656 233 -8150	-2698 233 -8150	-3266 233 -6150 -3304 233		-2333 233 -8150 -8150 -8150 -8150	-1455 233 -8150 -2491	-8150 -8150 -8150	4786 233 -8150 233 233
-1113 -500 -7106	-957 -500 -7108	-983 -500 -7108 -7108 -2680		-1462 -500 -7108 -7108 -7108	-2655 -560 -/108 -/108	-7106 -7106 -1045 -7198	-2159 -500 -7108 -500 -500
-149 -16	2003 - 149 - 16	-1171 -149 -16 -2594		-897 -149 -16 -1213 -149 -149 -149	-2171 -149 -16 -16 3134	-142 -56 -149 -16	-2631 -149 -16 -16 -16
272(M)	273(A)	274(L) - - 275(G)	276(G)	277(S) - - - - -	279(N) - - 280(A)	581(V)	282(L) - - 283(H)

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Table 12

<i></i>	/103300	و بسمر ر	136	PC1/US2011/	
290	291	293 294	295 296 297	298 290 300	
1782 -249	-2230 -249 -249	-2069 -249 -3320 -249	-1255 -249 -2749 -249 -1840 -249	-2308 -249 -249 -249 -249	-249
-1311	-2263 -294 -34(15) -294	-2286 -284 -3405 -294	-2146 -294 -3044 -294 -294 -294	-3125 -294 -294 -3215 -294 -294	-294
-369	56 -369 -2183 -369	524 -369 -369 -369	-2475 -369 -369 -369 -369 -369	-2506 -369 -369 -1866	-369
-1543	-2247 117 -1387 117	1840 117 -1387 117	-1550 117 117 -1208	-1284 117 117 117 117 117	117
-2389	-3604 359 -1216 358	-3044 359 -1216 359	-1377 359 359 359 -2087 359	-1030 -1030 -3593 -359 -566 -566 -358	359
-2613 96	-3579 96 -2944 96	-3251 96 -2944 96	-1000 98 96 -2153 96	-1331 96 -3843 96 96	96
-2281	-3105 45 -2798 45	-2334 45 -2798 45	-728 45 45 45	-377 -3628 -3628 -3628 -3628 -3628 -3628 -29	42
-3086 394	-3833 394 -2447 \$94	-3570 394 -2447 394	-22855 394 394 2992 2992	-1974 -1974 -3894 -3894 -3894 -2865	394
2785	-3962 275 -2236	-3451 275 275	2185 275 -1690 -275 -275 -275	1923 275 -3671 -275 -3671 -36 -365 -365	275
514 -720	523 -720 -720	3.09 -720 -720 -720	-2133 -720 -720 -720 -720	-2152 -720 -720 -1505 -720	-720
2271 466	25519 496 -3257 -466	335 -466 -3257 -466	-2770 -466 -2745 -466 -587 -466	-2936 -486 -486 -486 -486 -1869 -1869 -1869	465
-2808	-3843 210 -3028 -3028 -310	-3350 210 210 210	664 210 2301 -2011 -210 -210 -210		210
1373 -626 +	2176 -626 -526 * -526 *	3008 -626 * -5840 *	-2054 -626 * -2480 * -526 * -526 *	-2992 -5262 -526 -526 * -528 -526 *	-020
-1820 106 -1378	-3241 106 -1378* -1378 -1378*	-2801 106 -1378* -2735 106 -1378*	4320 106 -1376 -1376 -1378 -1378	-744 106 -1378 -3665 -3665 -1378 -366 -1378 -1378 -1378 -1378	1378
-32555 399 -701	-4326 396 -701 -701 399 -701	-701 -701 -701 -701	-1660 3999 -701 -701 -701 -701 -701 -701 -701 -701	-1011 -701 -701 -701 -701 -701 -701 -701	399
-251 -381 -11 15	-650 -381 -1115 -3267 -3267 -381	-877 -381 -1115 -3287 -381	-1816 -381 -381 -381 -381 -1115 -1115 -1320 -381 -381 -381	-3172 -3172 -1115 -1115 -1115 -2643 -381 -381	-381
-3164 43 -894	-4127 -4127 -894 -3040 -3040 -894	-3693 -364 -894 -3040 -3040	-470 43 43 43 43 -2131 -2131 -394 -5919 -5919 -594	1902 434 -894 -894 -894 -894 -3025	433
-3726 -333 -8150	-4640 233 -8150 -2846 233 -6150	-4176 233 -8150 -2846 -2846 -333 -8150	-362 233 233 -8150 -2328 -2338 -3388 -3388 -3388 -8150 -3388 -8150	311 233 -8150 -8150 -8150 1092 -8150 -3614	233 -8150
-1338 -500 -7108	-1873 -500 -7108 -1472 -630 -7108	-1507 -550 -5108 -7108 -1472 -500 -500	-2494 -500 -500 -7108 -500 -7108 -935 -935 -500 -7108	-2769 -500 -7105 -7105 -7105 -7108 -7108 -7108 -7108	-7108
-1623 -149 -16	-2333 -149 -16 -16 -149 -16	-1866 -149 -16 -16 -16 -16	-1450 -149 -149 -149 -149 -149 -149 -149 -149	-1243 -1499 -169 -169 -169 -1499 -166 -169 -166	-149
284(L)	285(L) - - - - - - -	287(M) - - 298(A)	239(H) 290(A) 291(I) 291(I)	292(G) - - 2293(V) - - 294(E) - - 294(E)	

Table 12

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		/10330				13'						11/025
302		303	304	305	306	307	30.8	309	310	314	312	313
-249		-1384 -249	-2864 -249	-249	349 -249	-2013 -249	-:404 -249	-791	-1573 -249	-1462 -249	-1961 -249	-2152 -249
-3137 -294		-1615 -294	-3864 -294	-3710 -294	-739	-2770	-2051 -294	-1522 -294	-2156	-2152 -294	-2248 -294	-2536
-1758		-50 -369	-3321 -369	-4046 -369	-2566	-2228 -369	-1367 -369	345 -369	-1684 -369	-1584 -369	591 -369	-369i
3168		-1369	-1913	-2938	-3420	-1028	1217	-1871	-614	·443	-1275	1858
1618 359		-2129 359	-1471 359	-2583	-3490 359	-848 359	-383 359	-2914 359	626 359	-369 358	-1394 359	-1527 359
-2214 96		-2133	-2283 96	-3232	-3645 96	-789 96	(670) 96	-3202 96	23311 96	1024 96	2765 95	-2587
-1916 45		1193 45	-760 45	-2039	-3350 45	2272	247	-2846 45	1632 45	342 45	-20 45	-2399
-1946 394		-2899 394	-2222 394	-3014 394	-3780 394	-18 49 394	-1553 394	-3506 304	-1687 394	-1525 394	-2245 394	-2663 394
-1538 275		-2422	-272	-1551 275	-3299	1814 275	-145 275	-3225 275	1161 275	-25 275	-046 275	-2305 275
-2043		508 -720	-3110 -720	-3956	-1900	-1769 -720	- <u>-</u> 324 -720	94 -720	-1151 -720	-1067 -720	-1473 -720	-695 -720
-2862		2102 466	-3720 -466	-4365 -466	-1801 -466	-2604 -466	-1793 -466	144 -466	-2020 -466	-1976 -466	-2199 -466	-1003 -466
2117		-2155 210	-1391 210	-2621 210	-4038	-255 210	214 210	-3351 210	444 210	282 210	2193 210	-2565 210
-2561 -826	*	1945 -628	-3846 -626	-4505 -626 *	-2315 -626	-2680 -626 *	-1755 -626	-626 -626	-2087 -626	-2025 -626	-2288 -626	470 -626
-2035 106	-1378	-1816 106	-1073 106 -1378*	-2197 106 -1378*	-1431 106 -1378	-505 106 -1378+	-1378 -1378	-2155 106 -1378*	-184 106 -1378*	1561 106 -1378*	-431 106 -1378*	-2324 106
-1236 399	101-	-3014 399	-1662 399 -701	-2466 399 701	-3545 399	-1535 399 - 701	-1406 399 -701	-3676 399 - 701	-1582 399 -701	- 1391 396	-2237 399 -701	-2234
	-1115	-528 -381 -115	-3883 -381 -115	-4140 -381 -1115	-1115 -1115	-2893 -381	-2053 -381	2027 -381 -1115	-2395 -381 -1115	-2275 -381 -1115	-2832 -381	-169 <u>2</u> -381
-2178 43	-894	-2576 43 -894	11999 43 -894	-1200 43 -894	-4232 43 -894	2170 43 894	135 43 891	-3667 43 -894	24 43 -894	1358 43 -894	-887 43 -894	-2832 43
-2204	-8150	-3144 233 -8150	3559 233 -6150	4018 233 -8150	-4026 233 -8150	205 233 -8150	631 233 -8150	-4077 233 -8150	-494 233 -8150	1622 233 -8150	-1793 233 -8150	-3099 233 84 50
	-7108	-1269 -500 -7108	-3682 -500 -7108	-3432 -500 -7108	-2776 -500 -7108	-2608 -500 -7108	-1687 -500 -7105	-1536 -500 -7108	-2015 -500 -7106	-1967 -500 -7108	-2315 -500 -7108	-1027 -520 7108
-149	-16	-1443 -149 -16	-1826 -149 -16	-2764 -149 -16	-3342 -149 -16	-1048 -149 -16	1083 -149 -16	-1915 -149 -16	-16j -149	387 - 149 16	-1460 -149 -16	-941 -149 -a
296(T) -		297(L)	298(D)	299(D)	300(F) -	301(Q)	302(R)	303(1)	304(R) -	305(D)	306(R) -	307(V) -

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Table 12

				· • • • • • • •	PCT/US2011/025
314	315 316	317 318	319 320 321	322 323	324 325
-4054 -249]	-707 -249 -249 -249	-249 -249 -2776 -249	-1830 -249 -249 -249 -249	-249 -3025 -249 -249	-1799 -249 -249
-3594	-1420 -294 -230 -234	-3091 -294 -3752	-1927 -294 -3001 -3001 -294	-294 -3310 -294 -294	-2234 -294 -441 -294
-4687	-130 -369 -369	-1624 -369 -3208 -369	-634 -389 -3831 -3831 -369	-369 -2413 -369 -4004	-1980 -369 -3671
3279	-1143 117 2344 117	-6622 117 -1833 117	-2350 117 -2536 117 -3279	-117 -1316 -117 -2931	-933 117
-3182 359	-1462 359 -3689 -3689	-540 359 -1416 369	-3550 359 -2604 -2604 -358	359 359 359 359	-952 359 -3418 359
3695	-1482 96 -3590	-2623 96 -2209	-3466 98 465 96 96	96 -2703 96 -3779 96	1344 96 .3341
378! 45	-1446 45 -3093 45	-2372 45 45 45 45	-2914 45 45 -1260 -1260 -3781	45 -2483 45 -3748 45	2273 45 45 45
394	-2482 394 -3862 394	-1943 394 394 394	-3754 394 3030 394	394 -2360 -2360 -3352 -3352	-1953 384
275	-1674 275 275 275 275	-1766 276 276 276 275	- 3948 - 275 - 2048 - 275 - 3481	275 -1973 -1973 -1973 -1973 	-562 275 -3027
-4165	591 720 587 -720	-1930 -720 -720 -720	2472 720 720 -720 -720 -4105	-720 -720 -720 -720	-13/3 -720 -726 -720
4490	-1027 -466 -466 -466	-2744 -456 -3641 -466	-3387 -3387 -3387 -466	-466 -3497 -466 -4671	-2192 -466 -466 -466
210	-1392 210 210 -3881 210	-2724 210 -1356 -1356 210	-3835 210 210 210 210 -3858	210 -2686 -3953 -3953	210 210 210 210
-629	-287 -626 * 1582 -626 *	-2398 -626 * -626 * -626 +	72 -626 * -3603 * -626 *	-626 -3294 -3294 -626 *	
-1378	2699 106 -1378* -3258 106 -1378*	-2337 196 -11378* -10665 10665 -1378*	-2920 106 -1376 -1570 106 -1376 -1378	106 -1378* -2474 106 -1378* -3462 -3462	-1378* -278 106 -1378* -1112 106
-2925 399 -701	-2221 -2221 -701 -701 -701	1022 399 -701 -701 -701 -701	4329 3999 7701 3999 3999 3999 3999 3999 3999 3999 2925	399 -701 -1640 399 -701 -701	-701 -1908 -701 -701
-4181 -381 -1115	-819 -381 -381 -588 -381 -381 -381	0872- 1186- 186- 186- 186-	1592 381 -381 -381 -381 -381 -1115 -4181	-381 -1115 -1115 -3185 -3185 -1115 -1115 -381	-1115 -2740 -381 -1115 -381 -381
3706 43 -394	-1824 43 -894 -4181 43 -894	-2573 43 -894 58 43 -894	4122 43 894 -2046 -2046 -3046 894 -3706	-894 -894 -3543 -3543 -3523 -3523 -3523 -3523	-894 -374 43 -894 -894 -3919
420 233 8150	-2097 -2033 -8150 -8150 -8150 -8150	-3012 233 -8150 -8150 -8150	4713 233 8150 -2481 -2481 -2481 -2481 -3481 -3420	233 	-8150 -1073 -333 -8150 -3701 -3701
-2878 -500 -7108	-1215 -500 -7108 -7108 -500 -500	4.168 -500 -7108 -7108 -7108 -7108	-2023 -500 -7108 -7108 -500 -7108	-500 -7108 -7108 -7108 -7108 -7108	-7108 -2257 -500 -7108 -500 -500
-2931 -149 -16	-1080 -149 -16 -2439 -149 -149	2157 -149 -16 -1732 -149 -149	-2477 -149 -149 -149 -149 -149 -149 -149	-149 -149 -149 -149 -149	-16) -149 -149 -149 -149 -149
308(P)	309(V) - - 310(L)	311(C) - - 312(D)	313(L) - - - - - - - - - - - - - - - - - - -	316(9)	319(Y)

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Table 12

7 2011	/103300	1 [] 1	F	139	, ,	[] I			T/US20:	[1/023.
326	327 328	329	330	331	332	333	334	335	336	337
-1933 -249	-586 -249 -1668	-249	-2214 -249	2920 -249	- : 469 -249	-1931 -249	-4222 -249	-4222	-2824 -249	-4064 - <u>2</u> 49
-2312	-951 -294 -2331	-3765 -3765	-2226 -294	123 -294	-2105	-2322 -294	-3668 -294	-3663 -294	-3265 -294	-3504 -294
- 369	154 369 -1734 -1734	-3226	-150	-2540	-1592 -369	-369	-4004	-4004 -369	2505 -369	-4087
-1558	712 117 117 117	-1850	-23555	-2055	-473 117	-1263	-2981	-2981	-1750	-3279
-2591	-1008 359 -613	-1429 359	-3698 359	-2761 359	-424 359	-2226 359	-2839	-2839	-3372 358	- 3182 359
-3011	-1411 96 -405	-2239	-3590 96	-2397 96	385 96	-2823 96	-3779	-3779	-3814 96	3695 96
-2779	-1:29 45 45 1857	45 172	-3091 45	-2191	352 45	-2585 45	-3748 45	.3748 45	-3693	-3781
-3326 394	-2028 394 -1704	-2211	-3885 394	-3499 394	-1570	-3093 384	-3352 394	-3352 394	-3877 394	12255
275	-1424 275 -163 -76	1366 275	-406%	-2279 275	275	-2718 275	-3320 275	-3320 275	-3731 275	-3491 275
-720	-720 -720	-3036	592 -720	-1948	-1056	-334 -720	4212	-720	-720	-720
-460	-210 -466 -466 -2127	-3652	2776 466	-2054 -466	-1954 -466	-615 -466	-4671 -466	-4671 -466	-603 -466	-466
210	-1396 210 81	-1361 210	-3854 210	-2463 210	210	-2867 210	-3953	-3953 210	-3840 210	-3859 210
ເ 9283 928	-626 + -2135 *	-3767	1510 -626 *	-2509 -628	-2013 -626 *	1765 -626 -	-626	-4761 -628	2375 -526 4	-4621 -626
-2635 106 -1378	-870 106 -1378* -317	-1378* -1378* -1378*	-3259 106 -1378+	4553 106 -1378-	-98 106 -1378*	-2406 106 -1378 -	-3462 106 -1378*	-3462 106 -1378 -	-3748 106 -1378*	-3468 106 -1378
-3357 399 -701	-1926 399 -701 -1495	-701 -701 -701	-4409 399 -701	-3449 399 -701	-1472 399 -701	-3026 339 -701	399 399	107- 1965	-4054 399 -701	-2925 399 -701
- 1103 -381 -1115	:656 -381 -115 -2416	-1115 -3744 -1115	-582 -381 -1115	826 381 1115	-2297 -381	-1376 -381 -1115	-4328 -381 -1115	-4323 -361 -1115	-1756 -381	-4181 -381 -1115
-894	-1673 43 -894 -894 1704	-894 90 -894	-4186 43 -894	-2926 43 -894	1025 43 -894	-3043 43 -894	-3623 43 -894	-3623 43 -894	-3970 43 -894	-3705 43 -894
-3829 233 -8150	-2256 233 -8150 -84	-8150 354.0 233 -8150	-4707 233 -8150	-2959 233 -8150	-342 233 -8150	-3509 233 -8150	-3304 233 -8150	-3304 233 -8150	-4331 233 -8150	-3420 233 -8150
-7108	-469 -500 -7108 -2094 -600	-7108 -3458 -500 -7108	-1983 -500 -7103	-2573 -560 -7108	-1957 -500 -7108	-1008 -500 -7108	-2690 -500 -7105	-2690 -500 -7108	-1302 -500 -7108	-2878 -500 -7108
-148	1225 -149 -16 -738 -149	-16 -1746 -149 -16	-2451 -149 -16	-2923 -149 -16	373 -149 -16	1739 -149 -16	-2594 -149 -16	-2594 -149 -16	-1758 -149 -16	2931 -149 -16
320(M)	321(M) - - 322(T)	323(D)	324(L)	325(H)	326(K) -	327(V)	328(G)	329(G) -	330(1)	33!(P) -

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Table 12

0 2011	/103300				140				PC	T/US201	11/0252
338	339	340	341	342	343	344	345	346	347	348	349
-1971 -249	-2677 -249	-1025 -249	-2968 -249	-249	-2220 -249	-220	-1581 -249	-1306 -249	-3156 -249	-794	-2214 -249
-2510 -294	-3074 -294	-2039	-3001 -294	-294	-2394	-2394	-2004 -294	-1934 -294	-3629	-1138 -294	-2226 -294
-1638 -369	698- 9828	-474 -369	-3331	-369	-1136 -369	-1136	1127 -369	32 2 -369	-3150 -369	52 -369	- 150
-659	-1917	-2274	2536	-1124	-2869	-7869	-805	-391	-1936	-875	-2355
-590	-2628 359	-3327 359	-2604 359	-1841	-3713 359	-3713 359	-851 359	-371 359	-1630 359	-1558 359	-3698 359
-901 96	-3437 96	-3171 96	-465 96	-2144	-3484 96	-3484 96	62 96	-244 96	-2576	-1815 96	3590 96
45	-3383 45	-2806 45	-1260 45	-1882 45	-3286 45	-3286 45	-26 45	2 <u>99</u> 45	-1424 45	-1557 45	-3091 45
-1796 394	-3399 394	-3671 394	-303H 394	-2699	-3797 394	-3797 394	-1900 394	-1530 394	-2480 394	-2453 394	-3665 394
-624 275	-3246 275	-3660	2043 275	275	-3935 275	-3935 275	029 275	275	-980 275	-1952 275	4069 275
-1426 -720	1076 720	4460 -720	-2339	1877	-31	-720	-1045	-815 -720	-720	516 -720	592 -720
-2256 -466	-1154 -466	1451 -466	-3387 -456	466	M30 486	466 466	-1600 -486	-1673 -466	-3792 -466	1793 -166	2775 466
-508 210	-3479 210	-3385 210	37.754 210	2270	-3734 210	-3734 210	2024 210	244 210	-1946 210	581 210	-3884 210
-2151 -625	403 -626 +	105 -626	-3603 -626 *	1805 -626 +	-541 -626 *	-541 -526	-1612 -626 -	-1618 -626 *	-3805 -626 *	1109 -526 +	1510 -626 +
-812 106 -1378-	-3231 106 -1378*	-2868 106 -1378*	-1570 106 -1378 *	-1255 106 -1378 *	-3165 106 -1378+	-3165 106 -1378	-407 106 -1378*	1528 106 -1378*	-1671 106 -1378*	-1269 106 -1378*	-3259 106 -1378 *
682 399 -701	-3050 399	-4051 399 -701		-2732 399 -701	-3803 899 -701	-3803 399 -701	-1703 3399 -701	-1415 399 -701	3263 107-	-2476 399 -701	-4409 399 -701
-2453 -381 -1115	-2037 -381 -115	-504 -115	-3743 -381 -1115	-117 -381 -115	-1033 -381 -:115	-1115 -1115	-2128 -381	-1926 -381	-1115	-1115	-58 <u>-</u> -381 -115
-492 43 -394	-3689 43 -394	-3834 43 -894	2646 43 -894	-2638 43 -894	-4103 43 894	-4103 43 -891	-360 43 -894	1008 43 -894	-690 43 -894	-2226 43 -894	-4166 43 -594
-730 233 -8150	-3750 233 -8150	-4343 233 -6150	2461 233 -8150	-3186 233 -8150	4231 233 -8150	-4231 233 -8150	-860 233 -8150	-275 233 -8150	1362 233 -8150	-2828 233 -8150	-4707 233 -8150
-1440 -500 -7106	-1603 -500 -7108	-1988 -500 -7108	-2961 -500 -7108	-974 -500 -7108	-2457 -500 -7108	-2457 -500 -7108	-1735 -500 -7108	-1636 -500 -7108	-2639 -500 -7108	-799 -500 -7108	-1983 -500 -7108
1795 -149 -16	-1771 -149 -16	-2355 -149 -16	-2620 -149 -16	-1187 -149 -16	-2871 -149 -16	-2871 -149 -16	-864 -149 -16	602 - 149 - 16	-1709 -149 -16	-942 -149 -16	-2451 -149 -16
332(Q)	333(V)	334(M) -	335(K) -	336(Y) -	337(L)	338(L)	339(K)	340(N) -	341(G)	342(F) -	343(L) -

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Table 12

	/103300		(1 I		14	1 []		[]		CT7US20	FT1/023
350	351	352	353	354	355	356	357	358	359	360	361
-1681 -249	-4222	-3552 -249	-1861 -249	-2224 -249	-3156 -249	-2248	-2858 -249	-4222 -249	-249	-3156 -249	-2369
-2397	3668 294	-3710	-2255	-2247	-3286 -294	-2657 -294	-3109 -294	-3668 -294	-2391	-3286 -294	-2634
-369	-4004 -369	-4046 -369	-1116	-369	-2197 -369	2269 -369	-1762 -369	-4604 -369	-2481	-2197 -369	369 1369
3356	-2981	-2938	-719	-2296	117	-1475	8428 117	-2981	-1477 117	3219	11733
-3291 359	2839 359	-2593	-732 359	-3647 359	-1463 359	-2770 359	905 359	-2839 359	-1592 358	-1463 358	-3164 359
2520	96 6276-	-3232 96	1534 96	-3586 96	-2753 96	-3335 96	-2260	-3779 96	1311 96	-2753 96	3547 96
-2835	-3748 45	-2039	-1147	-3098 45	-2708 45	-3129 45	-1991 45	-3748 45	2151 45	-2708 45	-3244
-3432 394	-3352 394	-3014 394	-1970 394	-3847 394	-2600 394	-3470 394	-1904 394	-3352 394	-2357 394	-2600 394	-3681 394
2856	-3320 275	-1551 275	-1351	-4023 275	-2311 275	-3224 275	-1571	-3320	-1035 275	-2311	3558 275
-720	-4212 -720	-3956 -720	-1086 -720	558 -720	-2612 -720	-413 -720	-2075 -720	-4212 -720	-1756 -720	-2612 -720	-66
-466	4671 466	-4365 -466	-1796 -466	21250 -466	-3105 -466	-617 -466	-2581 -466	-4671 -466	-2454 -466	-3105 -468	1520 466
210	-3953 210	-2621 210	-944 210	-3865 210	-2798 210	-3350	-2191 210	-3953 210	210	-2788 210	-3617 210
-4135	-4761 -623	-4505 -626	-1474 -626 *	1889 -626 *	-2698 -628	2612 -526 *	-2559 -626 *	-4761 -626	-2764 -528	-2698 -626 *	2139 -626
100 100 -1378	-3462 106 -1378	-2197 106 -1378*	-1302 196 -1378*	-3250 106 -1378 *	-2659 106 -1378 *	-2939 106 -1378*	-2090 106 -1378*	-3462 106 -1378 *	-444 1378*	-2659 106 -1378 *	3194 106
-3046 3999 -701	3748 399 899	-2466 399 -701	-1476 399 -701	-701	-1922 399 - 701	-3541 399 -701	-1245 399 -701	3788 399 -701	-701 396	-1922 399 -701	-3919 399
-2110 -381	-4328 -381 -115	-4140 -381	-1962 -381 -1115	-617 -381	-3163 -381 -115	-1524 -381 -115	-2904 -381 -115	-4328 -1115 -1115	-3336 -381 -1115	-3163 -381 -1115	-1235 -381
-2590 43 -394	-3623] 43 -894	-1200 43 -894	-1683 43 -894	-4155 43 -894	-2906 43 -894	-3563 43 -894	-2250 43 -894	-3623 43 -894	- 1008 43 -894	-2906 43 -894	-3816 43 -894
-2723 233 -8150	-3304 233 -8150	4018 233 -815C	-2162 233 -8150	-4674 233 -8150	-2755 233 -8150	4007 233 -8150	-2232 233 -8156	-3304 233 -8150	-2004 233 -8150	-2755 233 -8150	-4275 233 -8150
-3079 -500 -7106	2690 -500 -7108	-3432 -500 -7108	4452 -500 -7108	-1522 -500 -7108	-1674 -500 -7108	2972 -500 -7103	-979 -500 -7108	-2690 -500 -7108	-2632 -500 -7109	-1674 -500 -7102	-1339 -500 -7108
-3205 -149 -16	-2594 -149 -16	-2784 -149 -16	774 -149 -16	-2387 -149 -16	-1213 -149 -16	-1489 -149 -16	-364 -149 -16	-2594 -149 -16	-1716 -149 -16	-1213 -149 -16	-1771 -149 -16
344(H)	345(G)	346(D)	347(C) -	348(L.)	349(T)	350(C)	351(T) - -	352(G) -	353(K) -	354(1)	355(V)

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Table 12

	/103300						CT7US201	1/023
362	363	364	365	367 368	349	371	372	373
-249	-3385 -249	-1666 -249	-2099 -249 -3650 -249	-1397 -249 -684 -249	-1711 -249 -249 -2738	-1385 -249	-1616 -249	-521 -249
-3405 -294	-3563 -294	-2297	-2207 -294 -2296 -294	-2050 -294 -1058 -204	-2338 -294 -3765	-1882 -294	-294	-1136 -294
-2183	-3722 -369	1140	1281 -369 -1736 -369	-1409 -369 -369	-1524 -369 -3174 -3174	-1097	-1672 -369	- 568 - 369
-1357	-2750	-815	-2102 117 -670 117	793 117 117 117	-947 117 117 117	t;8. 1117	117	-381
-1216	2479 359	-757 359	-3239 359 -593 359	640 359 -1292 359	-882 359 -1346 -1346	-907 1589	-529 359	-539 359
-2944 96	-2560 96	-747 96	-3355 96 -251 96	-273 96 -1731	-1049 96 -2083 06	-360 96	-492 96	3609 96
-2798	-1842 45	-144 45	-29565 45 45 45	293 45 45 45	-363 45 45 45	88	1445 45	21 45
-2447	-2959 394	-1615 394	-3662 394 -1692 394	-1527 394 394 394	2037 394 394 304		394 394	775 394
-2234	-1531	275	-3673 276 -175 -175	-83 275 -1782 -275	427 275 1025 276	-558 -558 -275	-114 275	-440) 275
-2662 -720	-3555	-1191	467 -720 -1254 -720	-942 -720 -720 -720	-1245 -720 -720 -720	-649 -720	-1217 -720	-720
-3257 -466	4016	-1955 -466	-2116 -466 -2118 -466	-1825 -466 -222	187 -466 -466	1648 -466	-2086 -466	-894 -466
-3028 210	-2128 210	-265	-3561 210 1525 210	229 210 -1236 -1236 210	-1216 210	210	29 210	210
-2840	-626 +	-1711 -626		-1510 -626 + -526	-1787 -626 -3709	-1213 -628	-2089 -626	-740 -626
-2735 106 -1378	-2043 106 -1378*	-506 106 -1378	-3012 106 -1378 * -274 106 -1378 *	106 -1378+ -1113 -1113 -1378+	-687 106 -1378* -957	-1378* 505 -1378*	-265 106 -1378*	-183 106 -2249 -
-1726 399 -701	-2458 399 - 701	-1547 389 -701	-3965 399 -701 -701 -701	-1387 399 399 202 399 399 399	-1604 399 -701 -1599 399	-701 -1781 -101	-1436 399 -701	-:1496 399
-3287 -3287 -115	3966 381 115	-1115	-0/5 -381 -381 -115 -381 -381	-2092 -381 -1115 -581 -381	-2123 -381 -115 -3770 -381	-1115 -1115 -1115 -1115	-2354 -381	1406 -381
-3040 43 -394	3232 43 -894	1188 43 -894	-3584 43 -894 43 43 -804	214 43 -894 -2176 43 -891	-141 -141 -894 1256	-894 400 43 -894	203 43 -894	-304 43
-2846 235 -8150	-896 233 -8150	-96 -33 -315C	-4360 233 -8150 -175 233 -8150	1357 233 -8150 -8150 -8150 -8150	1681 233 -8150 3364 233	-8150 -633 -833 -8150	1139 233 -1646	-851 233 -7802
-1472 -500 -7106	-3308 -500 -7108	-1917 -500 -7138	-1779 -500 -7108 -7108 -500 -500	-1761 -500 -7108 -458 -500 -7108	-1912 -500 -7108 -7108 -500	-7108 -1646 -500 -7108	-2019 -500 -7108	-1009 -500 -8580
-149 -149 -16	-2641 -149 -16	-823 -149 -166		883 -149 -149 -149 -149	-922 -149 -1692 -1692	-16 -149 -16	-646 -149 -571	-422 -149 -23
356(A) - -	357(E)	358(N)	300(E)	361(H) 	363(P) - - 364(D)	- 365(Q)	366(P)	367(P)

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Table 12

	/103300		· · · · · · · ·		1 F 1 I	[] 1		17US20:	[1/025
374	375	376 377	378	379 380	381	382	383	384	385
-1752 -249	-2493 -249	-249 -249 -272	-249 -2770 -249	-2493 -2493 -249 -1128 -1128	-2151 -249	-1042 -249	-2021	-1791	-249
-2380	-3250 -294	-3077 -294 -3111	-294	-2877 -294 -294 -294	-2815	-294	-2817 -294	-2511 -294	-3260 -294
-1602 -369	-2426 -369	-3411 -369 -369	-369 -369	449 -369 -376 -369	-2050	-111 -369	-2243 -369	-1968 -369	-1913 -369
117	-1207	-2674 117 -1251	-1735	-2146 117 -507	-1014	-1508	-1011 117	-787	-014 117
-526 359	858 359	-2585 358 358		-3194 359 -634 359	012 359	-2381 359	207 359	-657 359	739 359
-746 96	-1669 96	-1665 96 1213	96 -3843 96	-3521 96 1074 96	-1131 96	-2616 96	-957 96	-522 96	-2406 96
-11 <u>8</u> 45	-644 45	4371 45 290	45 45 45	-3445 45 1171 45	45	-2297 45	1521 45	1005 45	-2114 45
1353 394	-1965 394	-3091 394 -1955	394 -3834 394	-3674 394 -1729 394	394	-3072 394	-1824 394	-1724 394	3274 394
-318 275	-395 275	-2107 275	275 -3671 275	-3562 275 275 275 -449	401 275	- <u>2</u> 822 275	-107 275	275	-1674 275
-1275	-2258 -720	-3081 -720 -2141	720	-693 -720 -720	-1786	558 -720	-1791 -720	-1475 -720	-2238 -720
-2126	-3050 466	-3469 -466 -2936	456 450 450 450	-559 -466 -374 -466	-2588 -466	1901 -466	-2636 -466	-2346 -466	-3009 -466
-246	-1033 210	-1565 210 1509	210 -3774 -210	-3581 210 212 212 210	-629 210	-2827 210	-344 210	1494 210	-2336 210
-2062 -629	-3045 -628	-3690 -526 * -3029	-626 -626	-1087 -526 -526	-2539	2071 -626	-620	-2416 -526	-2706 -626
-511 106 -1378	-982 106 -1378 -	-2079 106 -1378'	106 -1378* -3665 -106 -1378*	-3289 106 -1378 -1378 -106	-1376 - -794 -1378 -	-1926 106 -1378	-505 106 -1378	-341 106 -1378*	-2206 106
-1385 399 -701	3294 399 -701	-2661 399 -701	399 -701 -3979 -701	-3532 399 -701 -1659 399	-701 -1486 -701	-3245 399 -701	-1498 399 -701	-1487 399 -701	-1358 399 701
-2356 -381	-3222 -381 -115	-3251 -381 -1115 -3205	-1115 -1737 -1737 -381	-1676 -381 -115 -1467 -381	-1115 -2792 -381 -1115	-329 -381	-2008 -381 -1115	-2651 -381	-3058 -381
-70 43 -894	-100 43 -394	-1971 43 -894	+3 -894 -3921 -3921 -894	-3840 43 -894 -246	-891 1195 -894	-3197 - 43 -594	2310 43 -894	227 43 -894	-2301 43
1838 233 -8150	2141 233 -8150	-1886 233 -8150	233 -8150 -4281 -333 -8150	-3971 233 -8150 -847 233	-8150 -73 233 -8150	-3763 233 -8150	1844 233 -8150	1089 233 -6150	-2232 233 8150
-1668 -500 -7108	-2230 -500 -7108	-2904 -500 -7108	-7108 -7108 -1298 -500 -500	-1746 -500 -7108 -1354 -1354	-7108 -2031 -500 -7108	-1300 -500 -7106	-2640 -500 -7108	-2349 -500 -7108	-1116 -500
1472 -149 -16	-1044 -149 -16	-2562 -149 -16	-149 -16 -1738 -149 -16	-2091 -149 -16 -584 -584	-16 -149 -16	-1588 -149	-1024 -149 -16	-826 -149 -16	1032 -149 -16
368(D) -	369(G) -	370(G) - 371(D)	372(V)	373(I) - - 374(M)	- 375(P)	376(W)	377(E)	378(N)	379(P)

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Table 12

	/103300	, I [144				I/US201	<u> </u>
386	387	388	360	391 392	393	394	395	396	397
-1081 -249	2 888 -249	-1416 -249 -1777	-249 -249	-1652 -249 -2214 -249	-1135 -249	-249	-2220 249	2517 -249	-4222 -249
-1424	-2087	-2083 -294 -264	-294 -294	-2364 -294 -2226 -2326	-1579 -294	-3236	-2394	-1301 -294	-3668 -294
-369	-2075 -369	-1507 -369 -1581	-369 -369	-1924 -369 -150 -369	-339 -369 -	-369	-1136 -369	-1801 -369	-4004 -369
117	-1295	-414 117 1569	-2981	-992 117 -2355	-569	-1753	-2859	-1140	2081
-1591 359	-1411 359	-356 358	359 -2839	-881 359 -3698 359	-784 359	359	-3713 358	-1162 358	-2839
-1803 96	1983 96	-162 96	96 -3778 96	699- 96 95 95 95	-794 96	-389.3	-3484 96	114 96	-3779 96
-1580 45	-39 45	1252 45	45 -3748 45	-276 45 -3091 45	42	-3669 45	-328¢ 45	-167	-3748
-2507	-2258 394	547 394	394 -3352 394	1479 394 -3865 394	-1899 394	-3867 394	3797	-2165 394	- <u>3</u> 352 394
-1926 275	-949 275	-45 275	275 -3320 275	1362 275 -4069 -275	-823	-3/20	-3935	-808	-3320
-720	-1475 -720	-996 -720	-720	-1558 -720 592 -720	-297	÷12 -720	-31	-1282 -720	-4212 -720
1324 -466	-2195 -466	-1898 -166	-458 -4671 -466	-2338 466 466	-486	486	3130 -466	-1955 -466	-466
1084	2137 210	812 210 -23	210 -3953 -210	-296 210 -3384 210	210	210	-3734 210	2349 210	-3953 210
1452 -325	-2325	-1930 -526 *	-626 -4761 -626 *	-2320 -626 * 1510 * 526 *	-526 -526 -	* 9299 929	-541 -626	-2040 -626	-4761 -626
-1338 106 -1378	-420 106 -1378*	-89 106 -1378* -308	106 -1378* -3462 106 -1378*	3833 106 -1378 - -1378 - 106	-668 106 -1378 -	-3721 106 -1378 *	-3165 106 -1378	1794 106 -1378*	-1462 196
-2505 399 -701	-2254 399 -701	-1401 -1401 -701		-1600 399 -701 -701 399 -701	-1714 339 -701	-1038 399 -701	-3803 399 107-	-2105 399 -701	3783 399
-712 -381 -1115	2436 381 1115	-2196 -381 -115 -115	-381 -1115 -1115 -381 -381	-2264 -381 -1115 -582 -381	-1188 -381 -115	-1/30 -381 -115	-1115	-970 -381 -115	-4328 -381
-2129 43 -394	-887 43 -894	14071	43 -894 -3623 -3623 -894	-136 -136 -43 -4188 -43 -891	-721 43 -894	-390 - 43 -894	-4103 43 -894	-676 43 -894	-3623 43 -894
-2713 233 -8150	-1762 233 -8150	-206 233 -8150 1586	233 -8150 -334 -333 -8150	-200 233 233 -8150 -8150 -8150	-1196 233 -8150	-15C3 -8150	-4231 233 -8150	-1267 233 -8150	-3304 233 -8150
-773 -500 -7108	-2331 -500 -7108	-1890 -500 -7108	-500 -7108 -2690 -500 -500	-2039 -500 -7108 -7108 -500 -500	-1017 -500 -7108	-1300 -500 -7108	-2457 -500 -7108	-2115 -500 -7108	-2690 -500 -7108
-914 -149 -16	-1484 -149 -16	1256 -149 -16	-149 -16 -2594 -149 -18	-964 -149 -16 -16 -149 -149	1643 -149 -16	-1/00/ -149 -16	-2871 -149 -16	-1259 -149 -16	-2594 -149 -16
	381(Y) -	382(E) - - 383(G)	384(G)	385(H) - - - -	337(Q)	(1)064	389(L)	390(K) -	391(G)

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Table 12

0 2011	/103300				145				г. 	I/US201	1/0232
398	399	400	401		403	404	405	406	407	408	604
-249	-2220 -249	-3090 -249	-1565 -249 -2523	-248	-4222 -249	-3056 -249	-2677 -249	-3320 -249	-2988 -249	-2787	-249 -249
-294	-2394	-3331	-2214 -284 -3445	-294	-3668	-3306 -294	-3074 -294	-3405 -294	-3001	-3216 -294	-3217
-3392 -369	-1136 -369	-1844 -369	-1538 -369 -2852		-4004 -369	-1840 -369	369	-2183 -369	-3331 -369	2110 -369	-1824 -369
-2439	-2869	-746	677 117 -1527	117	-2981	-731	-1917	-1387	-2536 117	-1754	1398
-2224 359	-3713 359	936 359	1226 359 -1193	359	-2839 359	359	-2628 359	-1216 359	-2604 359	-3344 359	3 59
-2603	-3484 96	-2533 96	-143 96 -1711	96	96	-2471 96	-3437 96	-2944 96	-465 96	- <u>-</u> 3877 96	-2253
-2205 45	-3286 45	-2234 45	131 45 492	454	-3748 45	-2152	-3385	-2798 45	-1260 45	-3653	-1946
-2932 394	-3797 394	-1953 394	1198 394 -2057	394	-3352	-1940 394	-3399 394	-2447 394	-3039 394	-3859 394	-1946 394
3205 275	-3935 275	-166 9 275	-174 275	275	-3320	-1835	-3246	-2236	-2048	-3712 275	-1541
-3437 -720	-31	-2234 -720	-1115 -720 -2526	-720	-720	-2202 -720	-1676 -720	-2662 -720	-2839	-720	-2136 -720
3936 -466	<u>3130</u> 466	-3080 466	-1989 -466 -3256	-466	-4671 -466	-3057 -466	-1154 -466	-3257 -466	-3387	-556 -466	- <u>2970</u> -466
-2365 210	-3734 210	-2533 210	50 210 -9:54	210	-3953	-2439 210	-3479 210	-3028 210	210 210	-3814 210	-2157 210
-3943 -626	-541 -626 +	-2766 -526	-1968 -626 * -3354	-626	4761 -628 *	-2763 -626	403	-2840 -626 *	-3603 -628 *	-526 +	-2686 -626
-2267 106 -1376*	-3165 106 -1378*	-2295 106 -1378*	239 106 1378 * -843	106 - 1376*	-3462 106	-2235 105 -1378	-3231 106 -1378*	-2735 106 -1378	-1570 106 -1378	-3703 106 -1378*	-2073 106 1378 *
-701	-3605 396 -701	-1203 399 -701	-1396 399 -701	399 -701	3747 399 - 701	~1198 399 -701	-3050 -701	-1726 399 -701	-2791 396	-4027 399 -701	-1227 399 -701
-3334 -381	-1033 -1115	-3081 -381 -1115	-2248 -381 -1115 -3481	-115	4 328 -381 -1115	-3068 -381	-2037 -381 -1115	-3287 -381 -1115	-3743 -381 -1115	-1713 -381 -1115	-2989 -381 -115
-1748 43 -894	-4103 43 -894	-2561 43 -894	43 43 -894	43 -894	-3623 43 894	-2477 43 43	-3689 43 -894	-3040 43 -894	-2046 43 -894	-3954 43 -894	-2194 43 -894
-1458 233 -8150	-4231 233 -8150	-2489 233 -8150	-240 233 -8150 -8150	233 -8150	-3304 233 -8150	-2454 233 -8150	-3750 233 -8150	-2846 233 -8150	-2461 233 -8150	-4317 233 -8150	-2200 233 -8150
-2655 -500 -7106	-2457 -500 -7108	-934 -500 -7108	-1773 -500 -7108 -3230	-7108	-2690 -500 -7108	-932 -500 -7108	-1603 -500 -7108	-1472 -500 -7108	-2961 -500 -7108	-1312 -500 -7108	-931 -500 -7108
-2171 -149 -16	-2871 -149 -16	-149 -149 -16	-522 -149 -16 -16	-149	-2594 -149 -16	7843 -149 16	-1771 -149 -16	-16	-2620 -149 -16	-1761 -149 -16	-348 -149 -16
392(N)	393(L)	394(A) - -	395(E) - - 396(E)		397(G) -	398(A) -	399(V)	400(A)	401(K)	+02(J)	

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SUBSTITUTE SHEET (RULE 26)

Table 12

		\square			146	ΠΙΠ				Π
410	411	412	413	414	415	416 417	418	419	420	423
-4222 -249	-1151 -249	-2154	-1392 -249	-1769	1303 -249	552 -249 -1296	-2222	-2917 -249	-3320 -249	1960 249
-3868	-1514 -294	-2647	- 1962 284	-2347	-1016	-361 -294 -294	-254 -3668 -294	-3185	-3405 -294	-2249
-4604	-369	-2368	524 -369	-1876	-369	-317 -369 -369	-3091 -4604 -369	-1931 -369	-2183 -369	588 -369
-2981	-863	-1300	-516	-835	-360	-1220	-2961 117	1302	-1387	-1270
-2839	-1570 359	-1217 358	-518 359	-774 359	31 3 359	-1793 359 -468	-2839 359	-852 359	-1216 358	-1386 359
-3779 96	-1710 96	-3 96	-356 96	1519 96	475 96	-2075 96 -206	96	-2221 96	-2944 96	2627 96
3748	-1487 45	-166 45	73 45	5 5 5 5 7 5	-475 45	-1794 45 213	-3748 45	-2006	-2798 45	-17 45
-3352 394	-2501 394	-2117 394	-1658 394	394	-1807 394	-2692 394 394 -1801	-3352 -3352 394	386.0 394	-2447 394	-2240 394
-3520 275	-1841 275	-552 275	275	-330	600 275	-2038 275 -275	-3320 -3320	-1658 275	-2236	-940 275
-4212	94 -720	-1848 -720	844 720	-1375	178 -720	-720 -720	-720	-720	-2662 -720	-1473
4671	721 466	-2616 -466	-1655 -1665	-2210	-505 466	-445 -466 -1574	4671	-2928 -466	-3257 -466	-2200 466
210	1455 210	2980 210	632 210	241	-623 210	-2191 210 1092	-3953 - 210	-2128 210	-3028	2281 210
-626	1339 -626 *	-2762 -526 *	-1529 -626 *	-2268	-198 -626 *	-526 -526 -1449	-626 -626	-2631	-2840 -626 *	-2292 -626
-1378 ⁺	-1367 106 -1378*	-570 106 -1378	-307 106 -1378*	-35/ 106 -1378 *	-499 106 -1376	1767 106 -1378 -	-1378* -3462 -1378*	-2116 106 -1378 *	-2735 106 -1378*	-428 106
399 399 -701	-2491 399 -701	-1893 399 -701	1143 399 -701	-701	-1737 399 -701	-2683 -2683 -701 -1487	-701 399	-1453 396 -701	-1726 389 -701	-2232
-4328 -381 -1115	-827 -381 -115	-3108 -381 -115		-381 -381 -115	1053 -381 -115	2428 -381 -1115 -1115 -1830	-1115 -4328 -1115	-2996 -381 -1115	-3287 -381	-2834 -381
-3523 43 -894	-1976 43 -894	1824 43 -894	-36 43 -894	43 -894	-807 43 894		-594 -523 -523 -894	-2144 43 -894	-3040 43 -894	-67 <u>9</u> 43
-350 4 -8150	-2556 233 -8150	-447 233 -8150	-445 233 -8150	-359 233 -8150	-1376 233 -8150	-3022 233 -8150 -431		-2074 233 -8150	-2646 233 -8150	-1780 233
-7106	-809 -500 -7108	-2643 -500 -7108		-7108	-634 -500 -7108	-1082 -500 -7108 -7108			-1472 -590 -7108	-2316 -500
-149 -149	-917 -149 -16	-1386 -149 -16		-094 -149 -16	-419 -149 16		-169 -1491 -161		3438 - 149 - 16	-1454 -149
4.14(5)	405(V) -	4.066(K) -		+(m(r-)	(/)607	410(1) - +11(T)	412(G)	413(P) -	414(A)	<u>4.15(R)</u>

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SUBSTITUTE SHEET (RULE 26)

Table 12

	/103300	Π			14	7					п. П
422	423	424	425	426	427	428	429	430	431	432	433
- <u>2677</u> -249	349 -249	-2609	-2372 -249	-3385 -249	-1291 -249	-1537	-704 -249	-2058 -249	-1906 -249	-3226 -249	-2525 -249
-3074 -294	-739	-3585 -204	-3072 294	-3563 -294	-1 <u>397</u> -294	-2245) -294	-1068 -294	-2135 -294	-2668	-3415 -294	-2338
-369	-2566 -369	-369	-2226	-3722	-1502 369	-1662 -369	1209 -369	-369	-2073 -369	-1983 -369	2033
1111	-3420	-1623	-1044	-2750	-656	620 117	-529	-1961 117	-894 117	-848 117	-1742
-2828 359	-3490 359	-1253 359	2171 359	-2479	-611 359	713 359	-1090 359	-3183 359	-730 359	-654 359	-3260 359
3437 96	-3645 96	-1355 96	-1483 96	-2560 96	420 96	-349 96	-1091 96	-3313 96	-831 96	-2634 96	-3682 96
-3365 45	-3356 45	-535 45	-591 4 5	-1842 45	2163	272 45	-1459 45	-2896 45	-36 45	-2302	-3400 45
-3399 394	-3780 394	-2085 394	-1922 394	-2959 394	-1709 394	-1569 394	-2128 394	-3604 394	855 394	-2026 394	-3761 394
-3248 275	-3299 275	1292	-438	-1531 275	-210 275	-37 275	-1072 275	-3605 275	-107 275	-1722 275	-3632 275
-1076 -720	-1900	-2684 -720	-2046	-3655	-1054	-1157	250 -720	3085 -720	-1622 -720	-2379 -720	-217 -720
-1154 -466	-1801 -466	-3393 -466	-2867 -466	4016 466	-1842	-2058 -466	-334 -466	1204 -466	-2477 -466	-466	820 -466
210	-4038 210	-1050 210	-904 210	-2128 210	210	181 210	210	-3546 210	-243	-2626 210	-3705 210
403 -825	-2315 -628	-3513 -626 *	~2843 -626	-626 -626	-1824 -626	-2103 -526	-626 -626	2765 -626 -	-2527 -628	-2941 -526	2935 -626
-3231 106 -1378	-1431 106 -1378+	-879 106 -1378*	-939 106 -1378*	-2043 106 -1378*	-288 106 -1378 *	1830 106 -1378	-1091 106 -1378*	-2899 106 -1378*	-445 1378*	-2364 106 -1378*	-3383 106
-3050 399 -701	-3545 399 -701	-1583 389 -701	1600 399 -701	-2458 399 -701	-1534 399 -701	-1405 399 -701	-1966 399 -701	-3976 399 -701	-1477 399 -701	1898 399 -701	-3993 399
-2037 -381	4384 -381 -1115	-3613) -381] -115	-3045 -381 -1115	-3966 -381 -1115	917 381 115	-2349 -381 -1115	1204 -381 -1115	-659 -381 -1115	-2747 -361 -1115	-3226 -581	-1405
-3589 43 -894	-4232 43 -894	2447 43 -894	-177 -43 -894	3732 43 -894	2112 43 -894	1526 43 -891	-2107 43 -894	-3826 43 -894	2370 43 -894	-2539 43 -894	-3877
-3750 233 -8150	-4026 233 -8150	2573 233 -8150	1485 233 -8150	-896 233 -9150	- 199 233 -8150	1450 233 -8150	-2671 233 -8150	-4379 -8150	1356 233 -8150	-2418 233 -8150	-4307 233
-1603 -500 -7108	-2776 -500 -7108	-3426 -500 -7108	-1989 -500 -7108	-3308 -500 -7108	-1925 -500 -7108	-2048 -500 -7108	2678 -500 -7108	-1634 -500 -7106	-2447 -500 -7108	-1031 -500 -7102	-1325 -500
-1771 -149 -16	-5342 -149 -16	-1572 -149 -16	-879 -149 -16	-2641 -149 -16	-705 -149 16	-569 -149 -16	1626 -149 -16	-2042 -149 -16	412 -149 -16	2822 -149 -16	-1772
416(V)	417(F)	418(D) -	419(S)	420(E)	421(Q)	422(H) -	÷23(C)	424(M)	425(E)	426(A)	<u>4.27(l)</u>

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Table 12

	/103300		, , , , , , , , ,	148		r(CT/US2011/025
434	435	436 437	438	439 440	441	443	444 445
-1383 -249	-1643 -249	-2124 -249 -1607	-249 -2289 -249	-1366 -249 -249 -249	-4222 -249 -2780	-249 -2712 -249	-1724 -249 -249 -249
-1883 -294	-2298 -294		-294 -2517 -294	-1966 -294 -294	-3008 -294 -3756		-2084 -294 -3074 -3074
-1074	-1609 -369	-2245 -369 -1760		725 -369 -1923 -369	-4004 -369 -3213	-369 -369	-369 -369 -369
-816	-595	-1100 117 -619	117 -1976 117	-422 117 -894 117	-2981 117 -1838	117] -1726 117]	-1205 117 -1017 117
-909	1232 359	-915 358 -553	359 -3378 359	-396 359 359 359	-2839 359 359	359 359	-2074 358 -2628 359
-450 96	-561 96	866 96 1134	96 -35556 96	-48 96 95 95	-3779 96 -2218	96 -3767 96	-2622 96 3437 96
1405 45	52 45	-276 45 256	45	45 45 45 45 45	-3746 45 -760	45 -3556 -45	-2387 45 -3383 45
-1931 394	-1652 394	-1918 394 -1661	384 -3783 -394	-1550 394 1775 394	-3352 394 -2204	394 -3786 394	-2962 394 -3390
547 275	1001 275	-271 275	275 -3736 275	1165 275 275 -430	-3320 275	275 -3610 275	-2576 -275 -3246
825 -720	-1204 -720	-1860 -720	-720 145 -720	-905 -720 -720	-4212 -720 -3016	-720	-164 -720 -1076 -720
1884 -466	-2067 -466	-2675 -466 -2106	466 1100 -466	-1780 -466 -466 -466	-4671 -466 -466 -3647	-466 -582 -466	765 -466 -1154 -466
-135 210	-53 210	-463 210 2380	210 -3671 210	1157 210 210 210 210	-3953 210 -1363	210 -3706 -210	-2637 210 -3479 210
-1179 -626	-2041 -626	-2710 -526 *	+ + -626 -626	-1779 -628 -528 -526 -626	-4761 -626 - 3753	-626 * 1607 -628	845 -626 -403 *
525 106 -1378	-359 106 -1378	-658 106 -1378°	100 -1378 * -3253 106 -1378 *	1762 106 -1378 * -806 106 -1378 *	-3462 106 -1378* -1070	106 -1378* -3582 1378*	-2167 106 -1378* -1378* 106 1378*
-1769 399 -701	-1410 399 -701	1927 380 -701	-701 -701 -701	-1457 399 701 -1429 399 -701	372.7 399 -701 -1646	399 -701 -3894 396	-2888 399 -701 399 399
-1581 -381 -115	-2318 -381 -1115	-2921 -381 -1115 -2469	-1115 -1115 -1115 -381	-2078 -381 -381 -2714 -381	-4328 -381 -1115 -3737	-381 -1115 -1705 -361 -1115	-1164 -381 -1115 -1115 -381 -381
959 43 -894	949 43 -894	60 43 -894 588	-894 -3941 -3941 -3941 -894	171 43 43 43 43 894		43 -894 -3874 -3874 -894	-2911 -2911 -894 -3689 -3689
-575 233 -8150	-180 233 -8150	2583 233 -8150 785	-4380 -4380 -8150 -8150	-369 -369 -8150 -8150 -8150	-3304 233 -8150 3490	233 -8150 -4229 -8150	-3391 -3391 -8150 -3750 -3750 -3750
-1634 -500 -7108	-1826 -500 -7108	-2458 -500 -7108 -2117	-7108 -7108 -1582 -500 -7108	-1797 -500 -500 -7108 -500 -500 -7108		-500 -7108 -1302 -7108	-986 -500 -7108 -1603 -500
-875 -149 -16	1703 -149 -16	-1074 -149 -149 -166	-148 -16 -149 -149 -16	-460 -149 -166 -149 -16		-149 -16 -1721 -149 -16	594 -149 -1771 -149 -16 -149
	429(A) -	(1)(D) 			(<u>5)</u> (<u>5)</u> (<u>5)</u> (<u>1)</u> (<u>1)</u>) (<u>1)</u> (<u>1)</u> (<u>1)</u>) (<u>1)</u> (<u>1)</u>) (<u>1)</u> (<u>1)</u>) (<u></u>	- 437(V)	438(V) - - 439(V)

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Table 12

	/103300	1 kaana) 1 kaanad	149	,	PCT/US2	, , , , , , , , , , , , , , , , , , , ,
446	4 47 448	449 450	451 452	453 454	435 456	457
-249	-3185 -249 -249 -249	-790 -249 -249 -249	-4064 -249 -1717 -249	-4222 -249 -4222 -249	-4064 -249 -249	-2207
-2852 -294	-3096 -294 -198 -294	-1150 -294	-3594 -294 -2145 -294	-3068 -294 -3668 -3668	-3594 -3668 -294	-2513
-369	-3650 -369 -369 -369	1601 -369 -369 -369 -369	-4087 -369 536 -369	-4004 -369 -4004 -369	-4004 -369	-1398 -369
117	-2913 117 -1275	-621 117 -2981 117	-3279 117 -946	-2981 117 -2981	-3270 117 -2981 117	2552
-3243 358	-3026 359 -1518 358	-1311 359 -2839 359	-3182 359 -939 359	-2839 359 -2839 359	-3182 -359 -2839 359	-2911 359
-3679	4838 96 -1665 96	-1668 96 -3779 96	-3695 96 367	-3778 96 -3779	-3695 96 -3779 96	-2996 98
-3405 45	-1724 45 45 -1217 45	-1403 45 -3748 45	-3781 45 1339 1339	-3748 45 -3748 45	-3781 -3781 -3748 -3748	-3046
-3754 394	-3280 394 -2522 394	-2270 394 -3352 394	394 394 394	-3352 364 394	48886 394 3352 394	-3425
-3617 275	-2611 275 1178 275	-1700 276 -3520	-3491 275 -632 -632	-3320 275 -3320 275	-3491 275 -3320 -3320	-3293
-720	-3157 -720 -720 -720	261 -720 -4212 -720	-4165 -720 -720 -720	-4212 -720 -720	-4165 -720 -720 -720	5043 -720
400	-3631 466 -1211	679 -456 -4571 -4571	4490 466 1978	4671 466 4671	-4671 -4671 -4671	-596
210	-846 210 -1481 -1481	-1671 210 -3953 210	-3859 210 210 210	-3953 210 -3953 210	-3869 -3869 -3953 -3953	-3121 210
- -926 -	-3912 -626 * -626 *	1373 -626 * -4261 *	-4621 -626 -1970 -526	-4761 -626 -4761 -626	-4761 -528 -4761 *	-1547
-3377 106 -1378	-1968 -1968 -1378* -1378* -1378*	-1121 106 -1378 ⁴ -3462 106 -1375 ⁴	-3468 106 -1378 -357 -357 -1378	-3462 106 -1378* -3462 106	-3468 106 -1378* -3462 106 -1378*	-1378
399 399 -701	-2998 3999 -701 -701 389 -701	-2236 399 -701 399 399	-29255 3995 -701 -1917 -399 -701	3747 339 -701 399 3997 3997	-2925 399 399 -701 399 399	3105 399 701
-1115	-3796 -3796 -381 -381 -381 -115	-572 -381 -115 -115 -4328 -381	-4181 -381 -381 -381 -115	-4328 -381 -1115 -1115 -4328 -1115	-4181 4381 4328 4328	-1525 -381 -1115
-3367 43 -894	-2735 43 -894 -1608 -1608 -394	972 43 -894 -3623 43 -3623 -3623 -3623	-3706 43 43 43 43 43	-3823 43 -894 -894 -3623 -3623 -364	-3706 43 -894 -3023 -3023	3594 43 -594
-8150 -8150	-3518 233 -8150 -8150 -333 233 -8150	-2544 233 -8150 -3304 -3304 -8150	-3420 233 -8150 -1038 233 233 -8150	-3304 233 -8150 -3304 -8150	-3420 233 -8150 -3304 -3304 -8150	-3638 233 -8150
-1308 -500 -7106	-3022 -500 -7108 -1438 -600 -7108	2205 -500 -7108 -7108 -2680 -500 -500	-2878 -500 -7108 -2058 -500	-2690 -500 -7108 -7108 -7108	-2878 -500 -7108 -7108 -2690 -500 -7108	-2296 -500 -7108
-149 -169	-2957 -169 -16 -1321 -149 -16	-675 -149 -16 -2594 -149 -16	-2931 -149 -160 -160 -149 -149	-2594 -149 -149 -149 -149 -16	-2931 -149 -16 -16 -16 -149 -16	-2406 -149 -16
*40(I)	441(R) - - - - - -	443(C)	445(P) - - - - - -	-47(G) - - 448(G)	449(P) - - - - - - -	451(M)

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Table 12

	/103300		 				
458	459	460 461	462	463	465 456	467 468	469
-2613	- 3385 - 249	-2207 -249 -220	-249	-1878 -249 -2834 -249	-3025 -249 -1794 -249	-2265 -245 -2787 -2797	-4222 -249
-2815 -294	-3563 -294	-2513 -294 -2394	-2299	-2290 -294 -3139 -294	-3310 -294 -2145 -294	-2418 -294 -3216 -294	-3668 -294
-2593	-3722 -369	-1369 -369 -136	-369	-1231 -369 -1758 -369	-2413 -369 -592 -369	487 -369 -369 -369	-4004 -369
-1750	-2750	-2552 117 -2869	-587	-1674 117 117 117	-1316 117 	-2046 117 -1754 117	-2981
-1730 359	-2479 359	-3713 -3713	359	-1822 359 1543 359	359 359 -963	-3432 359 -3344 -3344	-2839 359
2095	-2560 96	-2996 96 -3484	470 96	-2129 96 -2218 96	-2703 95 -1932 96	-3557 96 -3877 96	3779 96
-1065	-1842 45	-3046 45 -3286	-160 45	-2061 45 -1923 45	-2463 45 -1713 45	-3171 45 -3653 45	-3748
3 23 8 394	-2959 394	-3425 394 -3797	-1721 	3430 394 394	-2300 394 -2188 394	-3788 394 -3859 394	-3352 394
-1624	-1531	-3293 -375 -3935	275 275	-2189 275 -1530 275	-1973 275 -1705 275	-3801 275 -3712 -3712	3320 275
-720	-3555 -720	-720	-1221	-841 -720 -720 -720	-2780 -720 -720	290 -720 -498 -720	-4212 -720
-2936	-4016 -466	-5965 -1666 -1866	-2068 -2068	1224 466 -2865 -2865	-466 -466 -968 -466	1619 -466 -466	-466 -466
450	-2128 210	-3121 210 -3734	210	-2063 210 -2125 -2125	-2680 210 -1858 210	-3723 210 	-3953 -210
-3000	-626	-1047 -528 -541	-1398	-1126 -626 * -526 *	-3294 -626 -626	526 + 526 + 526 + 526 + 526 + 526	-626
-1373 106 -1378	-2043 106 -1378	-2824 106 -1378 *	-1378 - -1378 - 106	-2022 108 -1378 1 -2041 106 -1378 1	-2474 106 -1378* -1706 -1378*	-3233 106 -1378 -3703 1378 *	-3462
-2242 399 -701	-2458 399 - 701	-3105 399 -701 -3803	-701 -1417 -701	-2358 399 -701 -1237 -701	-1640 339 -701 -701 -701	-4152 399 -701 399 399	66E
-381 -381	-3966 -381 -1115	-1525 -381 -115 -1033	-1115 -2319 -381	-1555 -381 -381 -381 -381 -115	-3185 -381 -1115 -1502 -1115	-1715 -381 -1115 -381 -381	-4328
- 154 8 4 3 - 894	32332 43 -394	-3594 -3594 -894 -8103	-894 -332 43 -804	-2380 43 -894 -2185 43 -894	-2543 -294 -894 -2144 -894	-3902 -394 -3954 -3954	-3623
-8150	-696 233 -8150	-3638 233 -6150 -4231	-8150 -763 233 -8150	-2514 233 -8150 -2208 233 -8150	-2333 233 233 -2150 -2150	-1461 233 -8150 -8150 233 233	-3304 233
-7108	-3308 -500 -7108	-2296 -550 -7108 -2457 -2457	-7108 -1481 -500 -7108	-1738 -500 -7106 -974 -500 -7105	-1462 -500 -7108 -986 -7108	-1859 -500 -7108 -1312 -600	-2690
-160% -149	-2641 -149 -16	-2406 -149 -16 -2871	-16 -149 -16	-1500 -149 -149 -149 -149 -149	-597 -149 -149 -16 -149	-2103 -149 -16 -1761 -149	-149
	453(E) -	454(M)	- -	457(P) - - - -	450(M)	461()) 	463(G)

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Table 12

0.11 0.11 <th< th=""><th>0.11 <th< th=""><th>1 1 1 1</th><th>2</th><th></th><th>5 V</th><th>1981</th><th>300</th><th>- Loui</th><th>1928</th><th>210</th><th>486</th><th>004</th><th>346</th><th></th><th>07-</th><th>3</th><th>000</th><th>2.4.5</th><th>100</th><th>2077-</th><th>-1657</th><th>4/0</th></th<></th></th<>	0.11 0.11 <th< th=""><th>1 1 1 1</th><th>2</th><th></th><th>5 V</th><th>1981</th><th>300</th><th>- Loui</th><th>1928</th><th>210</th><th>486</th><th>004</th><th>346</th><th></th><th>07-</th><th>3</th><th>000</th><th>2.4.5</th><th>100</th><th>2077-</th><th>-1657</th><th>4/0</th></th<>	1 1 1 1	2		5 V	1981	300	- Loui	1928	210	486	004	346		07-	3	000	2.4.5	100	2077-	-1657	4/0
301 303 <td>301 303 403<td></td><td>-7106</td><td>-8150</td><td>-994</td><td>-1115</td><td>-701</td><td>-1378</td><td></td><td>2</td><td>-60</td><td>h7 ;-</td><td>6/7</td><td>6AC</td><td><u>с</u>*</td><td>08</td><td>203</td><td></td><td>iaac-</td><td>-284</td><td>R47-</td><td></td></td>	301 303 403 <td></td> <td>-7106</td> <td>-8150</td> <td>-994</td> <td>-1115</td> <td>-701</td> <td>-1378</td> <td></td> <td>2</td> <td>-60</td> <td>h7 ;-</td> <td>6/7</td> <td>6AC</td> <td><u>с</u>*</td> <td>08</td> <td>203</td> <td></td> <td>iaac-</td> <td>-284</td> <td>R47-</td> <td></td>		-7106	-8150	-994	-1115	-701	-1378		2	-60	h7 ;-	6/7	6AC	<u>с</u> *	08	203		iaac-	-284	R47-	
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	1712 3.22 11.2 3.24 11.2 3.24 11.2 3.24 11.2 3.24 11.2 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 3.24 12.24 2.24 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 12.24 2.244 <t< td=""><td></td><td>-2690 -EVO</td><td>-3304</td><td>-3623</td><td>4328</td><td>3742</td><td>-3462</td><td>4761</td><td>-3953</td><td>4671</td><td>-42.12</td><td>-3320</td><td>-3352</td><td>-3748</td><td>-3779</td><td>-2839</td><td>-2981</td><td>-4004</td><td>-3668</td><td>-4222</td><td>471</td></t<>		-2690 -EVO	-3304	-3623	4328	3742	-3462	4761	-3953	4671	-42.12	-3320	-3352	-3748	-3779	-2839	-2981	-4004	-3668	-4222	471
1001 2001 1001 4001 1001 4001 1001 4001 1001 4001 1001 4001 1001 4001 1001 4001 1001 4001 1001 4001 1001 4001 10011 4001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 1001 <t< td=""><td>····································</td><td>191-</td><td>-7108</td><td>-8150</td><td>-394</td><td>-115</td><td>-701</td><td>-1378-</td><td></td><td></td><td>bot</td><td>677.</td><td>617</td><td>4.60</td><td></td><td>02</td><td>lace</td><td>1.1</td><td>1205-</td><td>146.7-</td><td>R17-</td><td></td></t<>	····································	191-	-7108	-8150	-394	-115	-701	-1378-			bot	677.	617	4.60		02	lace	1.1	1205-	146.7-	R 1 7-	
650 3244 4113 3561 3101 3564 4101 3564 4101 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 3564 210 366 220 366 220 3564 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 266 210 210 210 210 210 210 210 210 210 210 210 210 210 210 210 210 </td <td>7138 7133 7134 <th< td=""><td>-1699</td><td>-1807</td><td>-2268</td><td>-1925</td><td>-830</td><td>-2795</td><td>-1551</td><td>455</td><td>-1225</td><td>2530</td><td>06</td><td>-1958</td><td>-2845</td><td>1927</td><td>-1308</td><td>-2067</td><td>-1651</td><td>1</td><td>-1841</td><td>-1454</td><td>472</td></th<></td>	7138 7133 7134 <th< td=""><td>-1699</td><td>-1807</td><td>-2268</td><td>-1925</td><td>-830</td><td>-2795</td><td>-1551</td><td>455</td><td>-1225</td><td>2530</td><td>06</td><td>-1958</td><td>-2845</td><td>1927</td><td>-1308</td><td>-2067</td><td>-1651</td><td>1</td><td>-1841</td><td>-1454</td><td>472</td></th<>	-1699	-1807	-2268	-1925	-830	-2795	-1551	455	-1225	2530	06	-1958	-2845	1927	-1308	-2067	-1651	1	-1841	-1454	472
7/16 6/15 2/16 7/16 6/15 7/16 6/15 7/16 6/15 7/16 6/15 7/16 6/15 7/16 6/15 7/16 6/15 7/16 6/15 7/16 <th< td=""><td>$\begin{array}{c c c c c c c c c c c c c c c c c c c$</td><td>-149</td><td>-500</td><td>233</td><td>43</td><td>-381</td><td>369</td><td>106</td><td>-626</td><td>210</td><td>-466</td><td>-720</td><td>275</td><td>394</td><td>45</td><td>96</td><td>359</td><td>117</td><td></td><td>-204</td><td>-249</td><td></td></th<>	$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	-149	-500	233	43	-381	369	106	-626	210	-466	-720	275	394	45	96	359	117		-204	-249	
3500 3001 4023 4023 4024 4724 3723 3724 3784 4784 4773 3644 4733 3778 3784 4784 4773 3644 4733 3773 3764 3773 3764 3773 3764 3773 3764 3773 3764 3773 3764 3773 369 3773 <	300 300 300 300 300 300 300 300 300 300 300 300 400 <td>-16</td> <td>-7108</td> <td>-8150</td> <td>-894</td> <td>-115</td> <td>-701</td> <td>-1378</td> <td>*</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>1 </td> <td></td> <td></td> <td></td>	-16	-7108	-8150	-894	-115	-701	-1378	*										1			
7108 233 411 2101 2301 2	7108 734 434 7344 734 734 734 </td <td>594</td> <td>-2690</td> <td>-3304</td> <td>-3623</td> <td>4328</td> <td>2748</td> <td>3462</td> <td>-4761</td> <td>-3953</td> <td>-1673</td> <td>4212</td> <td>-3320</td> <td>33524</td> <td>3748</td> <td>0775</td> <td>.2830</td> <td>12081</td> <td>-4004</td> <td>196156</td> <td>6664</td> <td>173</td>	594	-2690	-3304	-3623	4328	2748	3462	-4761	-3953	-1673	4212	-3320	33524	3748	0775	.2830	12081	-4004	196156	6664	173
7/108 4/15 7/101 7/101 7/10	1/18 6154 594 1/15 2/11 2/18 6164 7/14 2/16	149	-500	233	43	-381	399	106	-626	210	456	720	275	394	5	96	359	117	696-	196-	676-	2
-3415 1711 -2713 -1446 -718 -2444 1734 -41 -560 -2344 1714 -2713 -1446 -731 -1446 -731 -1446 -731 -1433 -2442 -1701 -1334 -2412 -2413 -171 -2433 -2413 -2417 -2412 -1701 -2412 -2413	Artis T/TI T/TI <td>-16</td> <td>-7108</td> <td>-8150</td> <td>-894</td> <td>-115</td> <td>-701</td> <td>-1378*</td> <td>-</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>-</td> <td></td> <td></td> <td>2</td> <td></td>	-16	-7108	-8150	-894	-115	-701	-1378*	-									-			2	
-500 233 841 -360 103 -501 1378 -703 410 -704 -366 -210 -260 -213 -260 -213 -260 -213 -260 -213 -260 -213 -273 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -260 -274 -276 -275 -260 -274 -276 -275 -260 -276 -26	7108 510 233 443 366 117 366 136 136 236 <td>-853</td> <td>-2415</td> <td>2.05</td> <td>17171</td> <td>-2702</td> <td>-1468</td> <td>-378</td> <td>-2484</td> <td>1085</td> <td>-2417</td> <td>-1646</td> <td>194</td> <td>-1732</td> <td>41</td> <td>-6961</td> <td>6961</td> <td>-824]</td> <td></td> <td>-2594</td> <td>-(839]</td> <td>474</td>	-853	-2415	2.05	17171	-2702	-1468	-378	-2484	1085	-2417	-1646	194	-1732	41	-6961	6961	-824]		-2594	-(839]	474
7108 3150 994 1116 701 1288 701 418 701 418 701 418 701 418 701 417 708 417 708 710 710 711 708 711 708 711 708 711 708 713 701 7101 711 709 711 709 713 709 7101	7108 515 649 7116 701 470 703 415 701 416 701 406 770 406 701 703 416 701 406 701 406 703 406 703 </td <td>-149</td> <td>-500</td> <td>233</td> <td>43</td> <td>-381</td> <td>399</td> <td>106</td> <td>-626</td> <td>210</td> <td>466</td> <td>.720</td> <td>275</td> <td>394</td> <td>45</td> <td>96</td> <td>359</td> <td>117</td> <td>1</td> <td>-294</td> <td>-249</td> <td></td>	-149	-500	233	43	-381	399	106	-626	210	466	.720	275	394	45	96	359	117	1	-294	-249	
(178) -331 688 -2757 -1643 -370 -2472 1641 -2422 1711 -2422 1701 -3701 -3601 -3601 -3601 -3601 -3601 -3601 -3601 -2961 -2130 +75 -510 -5113 -701 1115 -701 1376 - - -360 -2130 -2691 -2130 -2691 -2303 -2991 -2991 -2991 -2991 -2991 -2991 -2991 -2991 -2101 -2492 477 - -2991 -2991 -2991 -2991 -2991 -2991 -2991 -2991 -2991 -2991 -2101 -2991 -2101 -2991 -2101 -2991 -2101 -2010 <td>-1780 -331 -689 -2757 -1643 -310 -2359 -1630 -2359 -2100 475 -500 2333 443 381 399 106 -239 -239 -299</td> <td>-16</td> <td>-7108</td> <td>-8150</td> <td>-894</td> <td>-115</td> <td>-701</td> <td>-1378 -</td> <td>*</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>-</td> <td>-</td> <td></td> <td>-</td> <td></td> <td></td>	-1780 -331 -689 -2757 -1643 -310 -2359 -1630 -2359 -2100 475 -500 2333 443 381 399 106 -239 -239 -299	-16	-7108	-8150	-894	-115	-701	-1378 -	*								-	-		-		
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7106 6150 894 111 1376 5701 2373 20301 20301 1268 2222 478 5101 2310 3569 1151 701 1376 2303 1261 22031 2619 22331 476 5101 2310 2569 260 2864 667 720 2303 459 951 394 459 394 471 369 2619 2334 471 369 2619 2334 471 369 2619 2334 471 369 2619 2334 471 369 2619 2334 471 369 2619 2334 471 369 2619 2334 471 369 2619 2334 471 369 2613 371 360 776 776 770 779 770 770 770 770 770 770 770 2613 2613 2613 2716 770 271 360 2761	····································	-149	-500	233	4	-381	396	106	-628	210	498	.720	275	394	45	98	359	117	-369	-294	-249	
352.3 370.2 340.8 167.0 354.9 317.1 369.1 128.8 2937 261.9 222.2 47.6 -500 233 -811.5 -701 -137.8 -501 -233.9 -186.8 -291.9 -293.9 -296.9 -294.9 -293.9 -296.9 -294.9	35.25 -37.02 -34.06 -167.0 -75.4 -31.61 -167.0 -25.4 -31.61 -167.0 -26.9 -21.03 -27.23 -28.75 -28.75 -28.19 -27.23 -28.95 -28.91 -27.23 -28.95 -28.91 -27.91 -28.91 -28.91 -28.91 -28.91 -27.91	-16	-7108	-8150	-894	-115	-701	-1378 +	*										-	-		
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-1632 -1434 -3975 -911 -4130 -2238 -2706 1451 244 -3773 -3785 3187 -3557 -3413 -2021 646 -2743 -2273 -500 233 43 -361 394 45 394 45 96 359 117 -369 -2844 -273 -7109 -8150 -894 -1115 -701 -1379* * 70 -466 -720 275 -394 45 96 359 117 -369 -284 -273 -1674 -2755 -2906 -1115 -701 -1379* * -275 -394 45 96 359 -2167 -3456 -2166 -2448 -204 -260 -2167 -243 -2446 -264 -2446 -266 -3156 -466 -264 -244 -246 -264 -246 -264 -246 -264 -244 -244 -244 -244 -244 -244 -244 -244 -244 -244 -244 -244 -244	-1632 -1434 -3975 -011 -4130 -2239 -2448 -2773 -3765 3187 -3557 -3413 -2021 646 -2449 -2773 -500 233 43 -361 394 106 -528 210 -466 -720 275 394 45 96 359 117 -369 -2449 -273 -500 233 43 -361 -701 -1378 * 1 -500 -594 -217 -249 -273 -249 -269 -219 </td <td>-16</td> <td>-7108</td> <td>-8150</td> <td>-894</td> <td>-1115</td> <td>-701</td> <td>-1378</td> <td>÷</td> <td></td>	-16	-7108	-8150	-894	-1115	-701	-1378	÷													
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Table 12

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-3779 96	4858 96	-3645 96	-2703	96 98	96 6276-	-2247 96	-3170 96	-3779	-3311 96	-3437 96	-3437 96
-3748 45	-1724 45	-3350 45	-2485 45	-3748 45	-3748 45	-1969 45	-2664 45	-3748 45	-2803 45	-3383	-3383 45
-3352 394	-3280 394	-3780) 394	-2360 394	-3952 394	-3352	-1958 394	-3621 394	-3352 394	-3633 394	-3399	-3399 394
3320 275	-2611 275	-3299 275	-1973 275	-3320	-3320	-1561 275	-2625	-3320	-3740 275	-3246	.3246 275
-720	-3157 -720	-1900	-2780	-4212 -720	-4212 -720	-2064 -720	-1973	-4212 -720	315R -720	-1076 -720	-1076 -720
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-3953 210	-846 210	-4038 210	-2656 210	-3953 210	-3953 210	-2170 210	-3541 210	-3953	-3649 210	-3479 210	-3479 210
-4761 -325	-3312 -626 *	-2315 -626 *	-3284	-4761 -626	-4761 -626 *	-2560 -626	-2526 -626 -	-4761 -626	67 -628 *	403 -526 *	403 -626
-3462 106 -1378	-1968 106 -1378*	-1431 106 -1378*	-2474 106 -1378 ⁴	-3462 106 -1378*	-3462 106 -1378*	-2074 106 -1378 *	3388 106 -1378 *	-3462 106 -1378*	-2676 106 -1378*	-3231 106 -1378*	-3231 106
37% 399 -701	-2996 399 - 701	-3545 399 -701	-:640 399 -701	3727 399 -701	3747 3999 701	-1242 399 -701	-3924 339 -701	3783 399	-4112 396 -701	-3050 -701	-3050 399 704
-4328 -381	-3796	-115 -115	-3185 -381	-4328 -381 -1115	-4328 -381 115	-2900 -381	1004 -381 -1115	-4328 -381 -1115	2387 -381 -1115	-2037 -381 -1115	-2037 -381
-3523 43 -394	-2735 43 -894	-4232 43 -894	-2543 43 -894	-3623 43 -894	-3623 43 -894	-2229 43 -894	4011 43 -894	-3623 43 -894	-3951 43 -894	-3689 43 -894	-3689 43
-304 233 -8150	-3318 233 -8150	-4026 233 -6150	-2333 233 -8150	-3304 233 -8150	-3304 233 -8150	-2225 -8150	-3941 233 -8150	-3394 233 -8150	-4536 233 -8150	-3750 233 -8150	-3750 233 8150
-2690 -500 -7108	-3022 -500 -7108	-2776 -500 -7108	-1462 -500 -7108	-2690 -500 -7103	-2690 -500 -7108	-976 -500 -7108	-2632 -500 -7108	-2690 -500 -7108	-1904 -500 -7108	-1603 -500 -7108	-1603 -500 7108
-169	-2957 -149 -16	-3342 -149 -16	-897 -149 -16	-2594 -149 -16	-2594 -149 -16	-359 -149 -16	-3402 -149 -16	-2594 -149 -16	-2322 -149 -16	-1771 -149 -16	-1771 -149 16
4/0(0)	477(R)	4 78(F) -		480(G) -	481(G) -	<u>482(1)</u>	433(Υ)	484(G)	485(M)	486(V) -	4 87(V)

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Table 12

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		,	154		1 [] 1 []		
906	507	510 510	244	512 513	514 515	516	517
-2493	-3228 -249 -249 -249 -249	-2219 -249 -1765	-249 -2169 -249	-3124 -249 -3552 -249	-585 -249 -2787	-249 -1783 -249	-2717 -249
-294	-3412 -294 -573 -294	-2536 -294	-294 -2390 -234	-3656 -294 -3710 -294	- <u>957</u> -294	-294 -294	-3126
-309	-1981 -369 -945 -369	-369 -369 -19:41	-369 -2385 -369	-3150 -369 -4046 -369	-369 2110 2110	-1400	1695 -369
117	-857 117 -2165	-1743 117 -863	117 1396 117	-1924 117 -2938	-413 117 -1754	117	-1764
-3194 359	-664 359 -2747 359	-2984 359 -858	359 -940 359	-1610 359 -2583 359	1079 359 -3344	358 -617 358	-3285 359
-3521 96	-2048 96 -2750 96	-3354 -96 1632	96 - 1192 96	-2539 96 -3232 96	-1131 96 -3877	96 -902	-3804 96
3445	-2327 45 -2438 45	-3094 45	45 -242 45	-1358 45 -2039	836 45 -3653	45 -368 45	-3576
-3674 394	-2034 -2034 -394 -3395 -3395	-3585 -3585 394 -1885	394 - 1905 394	-2456 394 -3014 394	-1937 394 -3859	394 -1787 394	-3822 394
-3562	-1739 275 -2786 -2786	-3467 -3467 -275	275 -145 275	-915 -275 -1051 -275	-1164 275 -3712	275 -526 275	-3672 275
-720	-2381 -720 -35 -35	-60 -720 -1356	-720 -720	-3119 -720 -5956	-120 -120	-720) -1203 -720	-720
-486	-3226 -466 -466	1254 -456 -2194	-466 -2792 -466	-3775 -466 -4565 -4565	566 -466 -556	-466 -19 <u>63</u> -466	-473 -466
-3581 210	-2650 -210 -2948 -2948	-3410 -3410 210 1444	210 -546 210	-1387 210 210 210 210	-9966 -9966 -3814	210 -472 -210	-3759 210
-928 -928	-2930 -626 * -542 -526	* 880 -626 * -2340	-526 -2549 -626	-3795 -526 +4505 -626	1519 -626 *	-626 -1791 -626	-626 -626
3230 106 -1378	-2380 106 -1378 * -1210 106	-1378* -3031 -1378* -252	106 -1376 -626 106 -1378	-1615 106 -1378* -2197 106	-687 -687 106 -1378	106 -1378* -709 1378*	-3619 106
-3532 399 -701	1051 399 -701 -3520 389	-701 -3714 -399 -701	399 -701 -701 -701	3339 3399 701 -2466 3399	-1879 399 -701	396 -701 -1425 -399 -701	-3967 399
-:679 -381 -1115	-3222 -381 -381 -381 -381 -381	-1115 -1174 -381 -3115 -1115	-381 -1115 -3046 -361	-3783 -381 -1115 -4140 -381	-468 -468 -381 -1115 -1115	-361 -1115 -2202 -381 -1115	- 1635 -381
-3540 43 -894	-2572 43 -394 -3400 43	-294 -3681 -3681 -894 -894	43 804 43 894	-614 43 -894 -1200 43	-12 <u>36</u> -12 <u>36</u> -894	-894 -894 1029 -894	-3923
-3971 233 -8150	-2445 233 -8150 -3711 233	-815C -815C -8150 -866	233 8150 2137 233 -8150	1591 233 8150 4010 233	-1819 -1819 -8150 -8150	233 8150 550 8150	-4283 233
-1746 -500 -7106	-1036 -500 -7108 -7108 -1892	-7108 -1387 -500 -7108	-7108 -7108 -2771 -500 -7108	-2684 -500 -7108 -3432 -3432 -500	-522 -520 -7108 -1312	-7108 -7108 -1467 -600 -7108	-1333 -500
-2091 -149 -16	3403 - 149 - 16 - 2239 - 149	-16 -149 -16 -982	-149 -16 -162 -149 -162	-1707 -149 -16 -16 -149 -149	-473 -149 -16	-149 -16 -16 -149 -16	-149
	501(A) 501(A) 502(L)	- 503(V) - 504(Q)		506(G) 	508(M)	- 510(1)	511(!)

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Table 12

	/103300	<u> </u>	,	155		PC1/US2	
518	519	520	521 522	523 524	525 526	527 528	529
-3552 -249	-2156 -249	-1397 -249	-1532 -249 -1663 -249	-905 -249 -2237 -249	-1430 -249 -1818 -249	-1678 -249 -2830 -249	2721 -249
-3710 -294	-2633	-2007] -294	-2113 -284 -2132 -294	-1379 -294 -294	-2110 -294 -1870 -294	-2382 -294 -3277 -294	-3057 -294
-4046 -389	-1703 -369	1267 -369	-1660 -369 -1755 -369	265 -369 -369	-1525 -369 570 -369	-1837 -369 -369 -369	-1755 -369
- <u>2938</u> 117	-910 [117	-585	-817 117 117 117	613 117 117 117	559 117 -2019 117	-677 117 117 117	-672
-2593 358	-809 359	-571 358	-540 359 -841 359	544 359 3594 359	530 359 -3095 359	-570 359 358 359	2362 359
-3232 96	-1061 96	-577 96	1058 96 1799 96	-565 -3570 96	-225 96 -3181 96	-401 96 96 96	-1999 96
-2039	204() 45	31 45	1043 45 45 45	-94 -3118 45	23 8 45 45 45	166 45 45 45	-1586 45
-3014 394	-1993 394	-1680 394	-1658 394 -1876 394	-1679 394 394	-1517 394 -3509 394	-1666 394 - 387 8 394	1826 394
275	-946 275	-223 275	1132 275 275	489 275 -3923 -395	-32 -3553 -3553	275 275 -3729 275	-1362 275
-3956	-1613 -720	-720	-1107 -720 -1204 -720	-172 -720 -720	-1016 -720 -720 -720	-1334 -720 -545 -720	-1932 -720
4365	-2337 -466	-1691 -466	-1937 -458 -456 -456	879 -466 -456 -456	-1921 -466 -466	-2217 -466 -466 -466 -466	- <u>2795</u> -466
-2621	-794 210	-86 210	210 210 210 210	-183 210 -3327 -3327 -3327	285 -3492 210	1719 210 -3841 210	-1788 210
-4505 -626	-2174 -626	-1558 -526	-2066	+623	-1853 -626 -626 -626 -626	-2279 -626 -626 * * 2623 -626	-2580
-2197 106 -1378	-1143 106 -1378*	26560 106 -1378*	-133 106 -1378* -253 106 106	-338 -1376 -1376 -3231 -3231 -1378	1434 106 -1378 -2674 106 -1378	1611 106 -1378 -3752 106	-1578 ⁴
-2466 399 -701	-1504 399 -701	168 399 -701	-1581 -701 -701 -1822 399 -101	-1583 399 -701 -701	-1389 -701 -701 -399 -701	-1466 399 -701 -701 399 399	-701
-4140 -381 -1115	-2506 -381 -115	-1883 -381 -115	-2376 -381 -1115 -1115 -2472 -381 -381	-1070 -381 -381 -708 -381 -1115	-2211 -381 -115 -1115 -381 -1115	-2518 -351 -1115 -1770 -381	-2867 -381
-1200 43 -394	-913 43 -894	66 43 -894	42 43 -894 -284 43 -384	4187 43 43 43 43 43 43	282 43 -894 -3796 43 -894	236 43 -894 -3968 43	-1625 -1625 -894
4016 233 -8150	-1036 233 -8150	1444 233 -6150	-546 233 -8150 -8150 -8150 -8150	-843 233 -8150 -8150 -8150 -8150	958 233 233 -8150 -8150 -8150	958 233 -8150 -8150 -4330 233 233 233	-2003 -2033 -8150
-3432 -500 -7106	-1451 -500 -7108	-1680 -520 -7108	-2036 -500 -7108 -2086 -500 -500		-1909 -500 -7108 -1743 -500 -7108	-2217 -500 -7109 -1297 -520 -520	-974 -500 -7108
-2784 -149 -16	2205 - 149 - 16	-615 -149 -16	-149 -149 -149 -149 -16		-477 -149 -169 -161 -1491 -161	-723 -149 -16 -1754 -149 -16	1545 -149 -16
512(D)	513(A)	514(H) -	515(K) - 516(N)	517(E) - - 518()) -	518(G) - - 520(L)	521(N) - - 522(V)	523(S)

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Table 12

) 2011/	103300			6		PC1/US201	. 1/025
530	531	532	534 535	536 537	538	539 540	541
-2816 -249	-2316 -249	-3385 -249 -249	-1728 -249 -1637 -249	-3185 -249 -249	-2374 -249	-1410 -249 -249	-1339 -249
-3821	-3177 -294	-3563 -264 -1924 -264	-2330 -294 -2150 -294	-3096 -294 -2421 -294		-2090 -294 -294	- 1996 - 294
369	-2569 -369	-3722 -369 -369 -382	-1691 -369 -1774 -369	-3650 -369 -369 -369	-1872 -369	-1492 -369 -369 -369	221 -369
-1855	-1314	-2750 117 -2226	-784 117 117 -724 117	-2913 117 -1619 117	-1103	562 117 117	595
-1125	-1()43 359	-2479 359 -3418 359	-717 352 -719 359	-3026 359 -1770 353	-954	-351 359 -1315 358	-303 359
-2201	-1403 96	-2560 96 -3418 96	96 96 2313 96	4558 96 3923	-1502 96	-228 96 -1627 96	-102 96
45	-347 45	-1842 45 -2902 45	-26 45 1328 45	-1724 45 1393 45	-934 45	1248 45 1369 45	1381 45
-2192 394	-1968 394	-2959 394 394	-1791 -1789 -1789	-3280 394 394 394 394	-2065	-1522 394 394	-1479 394
245	-161 275	-1531 275 -3825 275	-301 275 -379 275	-2611 275 	-809	1134 275 -1645 275	-33 275
-3029	-2196 -720	.3655 -720 -720	-1286 -720	-3157 -720 -1842 -720	-1873 -720	-720 -720 -720 -720	.809 -720
-3680 -466	-2979 -466	4016 466 466 456	-2103 -466 -2054 -466	-3631 -466 -2544 -466	-2591 -466	-1861 -466 -421	-1802 -466
-1322	-715 -716	-2128 210 -3758 210	954 210 210 210 229 210	210 210 2144 210	-1106	277 210 -1681 210	1168 210
-3788 -626	-3047 -626	-4105 -626 * -626 *	-2061 -626 + -2153 +	-3912 -626 -2356	-2333	-1909 -626 *	-1322 -626
-1031 106 -1378	-718 106 -1378*	-2043 106 -1378* -2649 106	-423 106 -1378 -1378 1632 106 1378	-1968 106 -1378 -431 -1378	-1218 106 -1378*	-109 106 -1378* -697 -697 -1378*	1530 106
-1642 399 -701	-1545 399 -701	-2458 -2458 -701 -701 -701	-15657 -15657 -701 -701	-2998 3999 -2562 3999 3995 -2562	-1546 399 -701	-1392 396 -2236 399 399	-1385 399
-3838 -381 -1115	-3223 -381 -115	-3966) -381 -1115 -1115 -381	-2385 -2381 -381 -381 -2504 -361	-3768 -381 -1115 -1115 -381 -381	-2769 -381 -1115	-381 -381 -381 -381 -381 -381	-209£
1869 43 -394	2896 43 -394	87322 43 -894 -894 -894 -894	938 43 -894 -146 -146 -146	-2735 43 -891 -1192 43 -694	857 43 -894	-227 43 -894 -2028 -2028	1284
2326 233 -8150	1944 233 -8150	-896 -8150 -8150 -8150 -8150 -8150		-3318 -3318 -8150 -8150 -8150 -8150	-553 233 -8150	-1/6 233 -8150 -2581 -2581 -8150	-274 233
-3649 -500 -7108	-2950 -500 -7108	-3308 -500 -7108 -7108 -7108 -500	-1980 -500 -7108 -7108 -2098 -500	-3022 -500 -7103 -7103 -7103 -7103	-1714 -500 -7108	-18/4 -500 -7108 -687 -500 -500 -7108	-1801
-1776 -149 -16	423 -149 -16	-2641] -149 -16 -2339 -149 -16	524 -149 -16 -16 -149 -149	-2957 -149 -1895 -1895 -149	293 <u>5</u> - 149 - 16]	-149 -16 -16 -16 -16 -16	-149
524(D)	525(E) -	526(E) - - 527(L) -	528(A) - - 529(R) -	530(R) - - 531(R)	532(A)	534(M)	535(H)

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Table 12

(170 (271 (281 (191 <th< th=""><th>1</th><th>537(P) - - - -</th><th>538(P) </th><th>541(Y) </th><th>543(R) 544(G)</th><th>545(V)</th><th>547(A) 547(A)</th><th></th></th<>	1	537(P) - - - -	538(P) 	541(Y) 	543(R) 544(G)	545(V)	547(A) 547(A)	
2227 17 1801 1916 1916 1916 1916 1916 1917 1917 1916 1917 1916 1917 1916 1917 1917 1917 1917 1917 1917 1917 1917 1917 1917 1917 1917 1917 1917 1916 1917 1916 1917 1916 1917 1916 1917 1916 1917 1916 1917 1916 1917 1917 1916 1917 1	-149 -16	-2931 -143 -324 -324 -149 -149	-1419 -1449 -1449 -1612 -1612 -1612 -16	712 -149 -527 -527 -149	-2957 -149 -169 -2594 -169	-1747 -149 -16	-28/1 -149 -16	-149
(-) (-) <td>-1/3/ -500 -7106</td> <td>-2878 -500 -7108 -924 -924 -6604</td> <td>-1017 -500 -7108 -7108 -500 -7108</td> <td>-796 -500 -7108 -7108 -7108</td> <td>-3022 -500 -7108 -7108 -7108</td> <td>-1296 -500 -7108</td> <td>-2457 -500 -7108 -890</td> <td>-500</td>	-1/3/ -500 -7106	-2878 -500 -7108 -924 -924 -6604	-1017 -500 -7108 -7108 -500 -7108	-796 -500 -7108 -7108 -7108	-3022 -500 -7108 -7108 -7108	-1296 -500 -7108	-2457 -500 -7108 -890	-500
· · · · · · · · · · · · · · · · · · ·	-02/ 233 -8150	-3420 233 -2368 -2368 -268 233 233 -7846		-2334 -233 -8150 1031 -8150 -8150	-3318 233 233 -8150 -3304 -3304 -3304 -332 -332 -8150	-4310 233 -8150	-4231 -8150 -1926	646
3101 113124 -1201 <th< td=""><td></td><td>-3708 -3708 -345 -546 -546 -594</td><td></td><td>-1283 43 894 -27 -27 -37 -37 -37</td><td>-2735 -2735 -894 -3623 -3623 -3623 -3623</td><td>-3948 43 -894</td><td>-4103 43 -894</td><td>1 J</td></th<>		-3708 -3708 -345 -546 -546 -594		-1283 43 894 -27 -27 -37 -37 -37	-2735 -2735 -894 -3623 -3623 -3623 -3623	-3948 43 -894	-4103 43 -894	1 J
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$	- 281 -381 -1115	-1115 -115 -115 -115 -115 -115	-1600 -381 -381 -2807 -281 -381	-370 -381 -381 -3815 -2315 -3815 -3815 -3815 -1115	-3796 -381 -381 -115 -4328 -381 -1115	-1758 -381 -1115	-1033 -381 -1115 -1803	100
····································	-1010 -701	-2925 -2925 -701 -701 -1356 -426 -426	-1586 -701 -2352 399	-2028 399 -701 -1379 399 -701	-29986 3999 -701 3999 -701	-4023 399 - 701	-3803 399 -701 1275	000
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$	106	-3468 -13/8* -13/8* -583 106 -1961*	-1411 106 -1378* -1378* -1378*	-986 -1376 -1376 -443 -1378	-1968 -1968 -1378 - -3462 -1378 -	-3716 106 -1378 *	-3165 -1378* -1378*	001
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	-626 -626			-143 -626 +2033 -626 *	-3812 -626 -626 -626 *	-523 -623	-526 -526 -1782	1000
····································	210			-1607 210 -151 -151	-3953 -3953	-3813 210	-3734 210	
2/75 3-34 5-31 -7-36 -5-74 -10.32 -3461 -457 -369 -3181 -369 -3182 -3294 -3461 -456 -3182 -3182 -3264 -3594 -2344 -3761 -3694 45 -96 -3182 -3177 -4687 -3594 -575 -3644 45 96 -359 -117 -369 -2344 -1364 3534 -451 96 -359 -117 -369 -234 -1364 3534 -1577 -947 -765 -763 -214 -275 -394 -45 96 -359 117 -369 -234 -275 -294 -1303 -1154 -765 -763 -294 -275 -294 45 96 -359 117 -369 -294 -275 394 -45 96 -359 117 -369 -294 -275 </td <td>-466</td> <td>-4490 -486 -1167 -466</td> <td>495 -486 665 -486</td> <td>-683 -466 -2081 -2081</td> <td>-3631 -466 -466</td> <td>-615 -466</td> <td>14694 202</td> <td>1007</td>	-466	-4490 -486 -1167 -466	495 -486 665 -486	-683 -466 -2081 -2081	-3631 -466 -466	-615 -466	14694 202	1007
	- <u>720</u>	-4165 -720 -720 -720	-720 -720 -720	-131 -720 -720	-5157 -720 -720 -720	-540	-720 -720	000
45 50 -572 -1245 -1956 -1954 3781 -3695 -3182 -3279 -4087 -3594 -294 45 96 359 117 -3699 -294 45 96 359 117 -369 -294 45 96 359 117 -369 -294 45 96 359 117 -369 -294 -51 96 359 117 -369 -294 -51 96 359 117 -369 -294 -51 96 359 117 -369 -294 -51 96 359 117 -369 -294 -51 96 359 117 -369 -294 -41 96 359 117 -369 -294 -417 369 -1743 -3241 -294 -417 369 3593 117 -369	275	-3491 275 -618 -575	-1364 275 -1051 -1051	-1587 275 -282 -282	-2011 275 -3320 275	-3705 275	-3935 275 .1316	515
501 -502 -17.2 -13.26 -11032 -369 3182 -359 117 -369 -294 -3695 3182 -3273 -40.67 -3594 -3294 -3695 359 117 -369 -294 -569 359 117 -369 -294 -560 359 117 -369 -294 -560 359 117 -369 -294 -1577 -367 117 -369 -294 -156 -1178 -771 1114 -965 -560 359 117 -369 -294 -666 -1178 -771 -1114 -294 -666 -1178 -117 -369 -294 -666 359 117 -369 -294 -666 359 117 -369 -294 -666 359 117 -369 -294 -3779 -369 -	394	48220 334 - 1660 394	315H 394 394	-2243 394 357 394	·3280 394 -3352 394	-3860 394	-3797 394 -1930	100
542 -1326 -1032 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -1545 -1771 114 -369 -359 117 -369 -294 -1178 -771 1114 -965 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 <td>40</td> <td>-3781 45 1324 45</td> <td>-1323 45 45</td> <td>-1363 45 45 45</td> <td>-1724 45 -3748 45</td> <td>-3670 45</td> <td>-3286 45 45</td> <td>1.020</td>	40	-3781 45 1324 45	-1323 45 45	-1363 45 45 45	-1724 45 -3748 45	-3670 45	-3286 45 45	1.020
-572 -1326 -1932 -117 -369 -294 -3279 -4087 -3594 -117 -369 -294 -117 -369 -294 -765 -763 -294 -117 -369 -294 -765 -763 -294 -765 -763 -294 -117 -369 -294 -711 114 -965 117 -369 -294 -771 1114 -965 -781 -117 -369 -781 -114 -366 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 -294 -117 -369 <td>88 98</td> <td>-369.5 96 -684 96</td> <td>-1577 96 96</td> <td>-1656 96 -850 96</td> <td>45.55 96 -3779 96</td> <td>-3267</td> <td>-3484 96 1674</td> <td>-1014</td>	88 98	-369.5 96 -684 96	-1577 96 96	-1656 96 -850 96	45.55 96 -3779 96	-3267	-3484 96 1674	-1014
-1326 -1932 -369 -294 -369 -294 -368 -369 -294 -368 -369 -294	359	-3182 359 -483 359	-347 359 -1545 352	-1178 359 1128 359	-3026 359 359 359	-3339 359	-3713 359 	1010
-1932 -294 -294 -294 -294 -294 -294 -294 -29	117	-3279 -117 -404 -117	[211] [211] [211] [211]	117 711 711	-2913 -2981	-1741	-2869	440-
	-1326	-4087 -369 -369 -369	-783 -369 -369 -369		-3850 -369 -4004 -369	369	-1136 -369 -369	702
-1394 -249 -249 -249 -249 -249 -249 -249 -2	-1932 -294	-3594 -294 -1703 -294	-2111 -294 -394	- 365 -234 -2321	-3096 -294 -3669 -3669	3252	-2394 -294 -287	19:2-
	-1394 -249	-4064 -249 -1242 -249	-1716 -249 -2014 -249	2423 -249 -1600 -249	-3165 -249 -222 -249	-2806	-2220 -249	n 9 : -
542 542 543 544 546 545 547 547 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548 543 548	542	543 544	545 546	547 548	549	551	552 883	566

Table 12

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3781 1570 3080 3387 3487 3486 <th< td=""></th<>
15/10 2000 3004 2504 3044 465 7004 2509 3014 2509 3014 2015 3014 2015 3014 2016 3016 <th< td=""></th<>
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210 -3031 -7203 -7204 -3033 -126 -3034 -3
3387 2839 2044 3039 1565 3039 1565 3031 <th< td=""></th<>
.2859 .0049 .1039 .1260 .3594 .451 .3604 .3594 .301
3033 1266 465 2304 177 -3304 -301 394 45 96 359 117 -369 -304 394 -27 96 359 117 -369 -304 394 -27 96 359 117 -369 294 394 -27 96 359 117 -369 294 394 -27 96 359 117 -369 294 394 -27 2778 -1276 117 -369 204 394 45 96 359 117 -369 204 304 -1777 -2773 459 -294 454 304 -1777 -369 117 -369 294 304 -165 359 117 -369 294 304 -17 369 -294 -294 -294 3054 45 96 359 117
·1266 ·465 ·2604 ·2536 ·3331 ·3001 45 96 359 ·117 ·369 ·294 2654 ·3451 ·329 ·349 ·264 ·294 45 96 ·359 ·117 ·369 ·294 2708 ·2944 ·1216 ·1387 ·2163 ·3005 -270 ·2708 ·2944 ·1216 ·1387 ·2043 45 96 ·359 ·117 ·369 ·294 -270 ·1621 ·1457 ·2043 ·2045 45 96 ·359 ·117 ·369 ·294 -1775 ·2708 ·1621 ·177 ·369 ·294 -1775 ·369 ·359 ·117 ·369 ·294 -1775 ·369 ·369 ·294 ·294 ·294 -1775 ·369 ·1717 ·369 ·294 ·294 -1330 ·117 ·369 ·294<
-465 2604 2536 3331 3001 96 359 117 -369 -264 96 359 117 -369 -264 96 359 117 -369 -264 96 359 117 -369 -294 237a -1216 -1387 -2183 -3405 96 359 117 -369 -294 96 359 117 -369 -294 96 359 117 -369 -294 96 359 117 -369 -294 96 359 117 -369 -294 96 359 117 -369 -294 96 359 117 -369 -294 96 359 117 -369 -294 97 359 117 -369 -294 96 359 117 -369 -294 96
2604 2536 3331 3001 359 117 -369 -294 -359 117 -369 -294 359 117 -369 -294 -1216 -1387 -2163 -3001 -1216 -1457 -269 -294 -1621 -1457 -369 -294 359 117 -369 -294 359 117 -369 -294 -1126 1070 2569 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 -359 117 -369 -294 <td< td=""></td<>
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-2001 -294 -294 -294 -294 -214 -214 -294 -294 -294 -294 -294 -294 -294 -29
-2983 -249 -249 -249 -3320 -249 -249 -249 -249 -249 -249 -249 -249

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Table 12

		İ															
566			567			072	900				5 69			570			
-2386	-248		-2677	-249		3466	0010-	-249			-3552	-249		-23U			
-3099	-294		-3074	-294		0000	10:070-	-264			-3710	-294		-714	<u>,</u>		
-1463	-369		3636	-369		0407	1817-	-369			-4046	-369		336	.*		
-799	117		-1917	117		100 100 100	20. 30	117			-2038	117		-465	¥		
-861	359		-2628	359			-1403	358			-2593	359		-1057	*		
-2727	96		-3437	96		- Justo	-2103	96			-3232	96		-1362	•		
-2547	4 10 4		-3383	45		0100	-2/08	45			-2039	LC T		-1067	•		
-2044	394		-3399	394		- Prove	10097-	394			-3014	394		-2030	*		
-1927	2753		-3246	275			152-	275			-1551	275		107.9	,		
-1924	-720		-1076	-720		191.00	-2612	-720			-3356	-720		561	<u>.</u>		
-2528	466		-1154	466		1-25.2	-3100	466			-4365	-466		952	•		
-2882	210		-3479	210		120-12	-2/88	210			-2621	210		-1346	k	o	
-2065	-626	<u>+</u>	403	-626	*	0000	2692-	-526	×		-4505	-626	*	1247	*		
-2462	:06	-1378	-3231	106	-1378*	1.	RC97-	106	-1378		-2197	106	-1378 *	-744	•	7	
-: 303	399	-701	-30501		107-	10000	-1922	399	-701		-2466	399	-701	-2001	•	*	
-2739	-381	-1115	-2037	-381	-1115	11222	-3163	-381	-115		-4140	-381	-:115	12116	•	*	
-3158	43	-394	-3689	43	-894		-250 to	Et.	-894		-1200	43	-894	-1627	•	*	
-6185 -	233	-8150	-3750	233	-8150		-2/25	233	-8150		4018	233	-7757	-2202	*	*	
3772	-500	-7108	-1603	-500	-7108		-16/4	-500	-7108		-3432	-500	-6715	-445	*	*	
2804	-149	-16	-1771	- 149	-16		-1213	-149	-16		-2764	-149	-21	-525		*	
560(C)	-		561(V)		-		562(1)	-	-	S	563(D)	-		564(F)	-	*	

Table 12

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WHAT IS CLAIMED IS:

1. A recombinant host cell comprising:

(a) at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and

(b) (i) at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis; and/or

(ii) at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis.

2. The recombinant host cell of claim 1, wherein said at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity:

(a) comprises a high copy number plasmid or a plasmid with a copy number that can be regulated; or

(b) is integrated at least once in the recombinant host cell DNA.

3. A recombinant host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity wherein said at least one heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated.

4. A recombinant host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity wherein said at least one heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.

5. The recombinant host cell of claim 1 or 2, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by a gene selected from the group consisting of the genes in Tables 8 and 9.

6. The recombinant host cell of claim 1 or 2, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by a gene selected from the group consisting of the genes in Table 7.

7. The recombinant host cell of claim 5 or 6, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by a gene selected from the group consisting of AFT1, AFT2, FRA2, GRX3, CCC1, and combinations thereof.

8. The recombinant host cell of claim 7, wherein said polypeptide is encoded by a polynucleotide that is constitutive mutant.

9. The recombinant host cell of claim 8, wherein said constitutive mutant is selected from the group consisting of AFT1 L99A, AFT1 L102A, AFT1 C291F, AFT1 C293F, and combinations thereof.

10. The recombinant host cell of claim 1 or 2, wherein said polypeptide affecting Fe-S cluster biosynthesis is AFT1, AFT2, FRA2, GRX3, or CCC1.

11. The recombinant host cell of claim 1 or 2, wherein said polypeptide affecting Fe-S cluster biosynthesis is AFT1, AFT2, FRA2, or CCC1.

12. The recombinant host cell of claim 1 or 2, wherein the at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of FRA2, GRX3, CCC1, and combinations thereof.

13. The recombinant host cell of claim 1 or 2, wherein the at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis is selected from the group consisting of AFT1, AFT2, and combinations thereof.

14. The recombinant host cell of claim 1, wherein said at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is expressed in multiple copies.

15. The recombinant host cell of claim 14, wherein said at least one heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated.

16. The recombinant host cell of claim 14, wherein said at least one heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.

17. The recombinant host cell of any one of claims 1,2, or 5-16, wherein said Fe-S cluster biosynthesis is increased compared to a recombinant host cell having endogenous Fe-S cluster biosynthesis.

18. The recombinant host cell of any one of claims 1-17, wherein said host cell is a yeast host cell.

19. The recombinant host cell of claim 18, wherein said yeast host cell is selected from the group consisting of Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia and Pichia.

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20. The recombinant host cell of any one of claims 1-19, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in the cytosol of the host cell.

21. The recombinant host cell of any one of claims 1-20, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of < 10-5 wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the Streptococcus mutans DHAD enzyme corresponding to SEQ ID NO:168.

22. The recombinant host cell of any one of claims 1-21, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence with at least about 90% identity to SEQ ID NO: 168 or SEQ ID NO: 232.

23. The recombinant host cell of any one of claims 1-22, wherein said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of:

(a) greater than about 5-fold with respect to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity;

(b) greater than about 8-fold with respect to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and

(c) greater than about 10-fold with respect to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity.

24. The recombinant host cell of any one of claims 1-22, wherein said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of:

- (a) greater than about 0.25 U/mg;
- (b) greater than about 0.3 U/mg;
- (c) greater than about 0.5 U/mg;
- (d) greater than about 1.0 U/mg;
- (e) greater than about 1.5 U/mg;
- (f) greater than about 2.0 U/mg;
- (g) greater than about 3.0 U/mg;

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- (h) greater than about 4.0 U/mg;
- (i) greater than about 5.0 U/mg;
- (j) greater than about 6.0 U/mg;
- (k) greater than about 7.0 U/mg;
- (1) greater than about 8.0 U/mg;
- (m) greater than about 9.0 U/mg;
- (n) greater than about 10.0 U/mg;
- (o) greater than about 20.0 U/mg; and
- (p) greater than about 50.0 U/mg.

25. The recombinant host cell of any one of claims 1-24, wherein said recombinant host cell produces isobutanol.

26. The recombinant host cell of claim 25, wherein said recombinant host cell comprises an isobutanol biosynthetic pathway.

27. A method of making a product comprising:

(a) providing the recombinant host cell of any one of claims 1-24;

(b) contacting the recombinant host cell of (a) with a fermentable carbon substrate in a fermentation medium under conditions wherein said product is produced;

wherein the product is selected from the group consisting of branched chain amino acids, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol, and combinations thereof.

28. A method of making isobutanol comprising:

(a) providing the recombinant host cell of any one of claims 1-24;

(b) contacting the recombinant host cell of (a) with a fermentable carbon substrate in a fermentation medium under conditions wherein isobutanol is produced.

29. A method for the conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate comprising:

(a) providing the recombinant host of any one of claims 1-24; and

(b) growing the recombinant host cell of (a) under conditions where the 2,3dihydroxyisovalerate is converted to α -ketoisovalerate,

wherein 2,3-dihydroxyisovalerate is converted to α -ketoisovalerate.

30. A method for increasing the specific activity of a heterologous polypeptide having dihydroxy-acid dehydratase activity in a recombinant host cell comprising:

(a) providing a recombinant host cell of any one of claims 1-24; and

(b) growing the recombinant host cell of (a) under conditions whereby the heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in functional form having a specific activity greater than the same host cell lacking said heterologous polypeptide.

31. A method for increasing the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising:

(a) providing a recombinant host cell of any one of claims 3-24; and

(b) growing the recombinant host cell of (a) under conditions whereby the flux in the Fe-S cluster biosynthesis pathway in the host cell is increased.

32. A method of increasing the activity of an Fe-S cluster requiring protein in a recombinant host cell comprising:

(a) providing a recombinant host cell comprising an Fe-S cluster requiring protein;

(b) changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis in said host cell; and

(c) growing the recombinant host cell of (b) under conditions whereby the activity of the Fe-S cluster requiring protein is increased.

33. The method of claim 32, wherein said increase in activity is an amount selected from the group consisting of:

(a) greater than about 10%;

(b) greater than about 20%;

(c) greater than about 30%;

- (d) greater than about 40%;
- (e) greater than about 50%;
- (f) greater than about 60%;
- (g) greater than about 70%;
- (h) greater than about 80%;
- (i) greater than about 90%; and

(j) greater than about 95%.

34. The method of claim 32, wherein said increase in activity is an amount selected from the group consisting of:

(a) greater than about 5-fold;

- (b) greater than about 8-fold; and
- (c) greater than about 10-fold.

35. A method for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising:

(a) changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis;

(b) measuring the activity of a heterologous Fe-S cluster requiring protein; and

(c) comparing the activity of the heterologous Fe-S cluster requiring protein measured in the presence of the changed expression or activity of a polypeptide of step (a) to the activity of the heterologous Fe-S cluster requiring protein measured in the absence of the changed expression or activity of a polypeptide of step (a),

wherein an increase in the activity of the heterologous Fe-S cluster requiring protein indicates an increase in the flux in said Fe-S cluster biosynthesis pathway.

36. A method for identifying polypeptides that increase the flux in an Fe-S cluster biosynthesis pathway in a host cell comprising:

(a) changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis;

(b) measuring the activity of a polypeptide having dihydroxy-acid dehydratase activity; and

(c) comparing the activity of the polypeptide having dihydroxy-acid dehydratase activity measured in the presence of the change in expression or activity of a polypeptide of step (a) to the activity of the polypeptide having dihydroxy-acid dehydratase activity measured in the absence of the change in expression or activity of a polypeptide of step (a),

wherein an increase in the activity of the polypeptide having dihydroxy-acid dehydratase activity indicates an increase in the flux in said Fe-S cluster biosynthesis pathway.

37. The method of any one of claims 30-36, wherein said changing the expression or activity of a polypeptide affecting Fe-S cluster biosynthesis comprises deleting, mutating, substituting, expressing, up-regulating, down-regulating, altering the cellular location, altering the state of the protein, and/or adding a cofactor.

38. The method of any one of claims 32-37, wherein the Fe-S cluster requiring protein has dihydroxy-acid dehydratase activity and wherein said Fe-S cluster requiring protein having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of < 10-5 wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the Streptococcus mutans DHAD enzyme corresponding to SEQ ID NO:168.

39. The method of any one of claims 32-38, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by a gene selected from the group consisting of AFT1, AFT2, FRA2, GRX3, CCC1, and combinations thereof.

40. A recombinant host cell comprising at least one polynucleotide encoding a polypeptide identified by the methods of any one of claims 35-37.

41. The recombinant host cell of claim 40, wherein said host cell further comprises at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity.

42. The recombinant host cell of claim 41, wherein said heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is expressed in multiple copies.

43. The recombinant host cell of claim 41, wherein said heterologous polynucleotide comprises a high copy number plasmid or a plasmid with a copy number that can be regulated.

44. The recombinant host cell of claim 41, wherein said heterologous polynucleotide is integrated at least once in the recombinant host cell DNA.

45. The method of claim 35 or 36, wherein said host cell is a yeast host cell.

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46. The method of claim 45, wherein said yeast host cell is selected from the group consisting of Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia, and Pichia.

47. The method of any one of claims 28-39, wherein said host cell is a yeast host cell.

48. The method of claim 47, wherein said yeast host cell is selected from the group consisting of Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia, and Pichia.

49. The recombinant host cell of any one of claims 40-44, wherein said recombinant host cell is a yeast host cell.

50. The recombinant host cell of claim 49, wherein said yeast host cell is selected from the group consisting of Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia, and Pichia.

51. The recombinant host cell of any one of claims 40-44 or 49-50, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity is expressed in the cytosol of the host cell.

52. The recombinant host cell of any one of claims 40-44 or 49-50, wherein said heterologous polypeptide having dihydroxy-acid dehydratase activity has an amino acid sequence that matches the Profile HMM of Table 12 with an E value of < 10-5 wherein the polypeptide further comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the Streptococcus mutans DHAD enzyme corresponding to SEQ ID NO:168.

53. The recombinant host cell of any one of claims 40-44 or 49-50, wherein said recombinant host cell produces a product selected from the group consisting of branched chain amino acids, pantothenic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol, and combinations thereof.

54. The recombinant host cell of claim 53, wherein said recombinant host cell produces isobutanol.

55. The recombinant host cell of claim 54, wherein said recombinant host cell comprises an isobutanol biosynthetic pathway.

56. The recombinant host cell of any one of claims 1-22, wherein said polypeptide having dihydroxy-acid dehydratase activity has a specific activity selected from the group consisting of:

(a) greater than about 3-fold with respect to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and

(b) greater than about 6-fold with respect to a control host cell comprising at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity.

57. The method of claim 32, wherein said increase in activity is an amount selected from the group consisting of:

(a) greater than about 3-fold; and

(b) greater than about 6-fold.

58. The recombinant host cell of any one of claims 1-22, 40-44, or 49-50, wherein monomers of said polypeptide having dihydroxy-acid dehydratase activity have an Fe-S cluster loading selected from the group consisting of:

- (a) at least about 10%;
- (b) at least about 15%;
- (c) at least about 20%;
- (d) at least about 25%;
- (e) at least about 30%;
- (f) at least about 35%;
- (g) at least about 40%;
- (h) at least about 45%;
- (i) at least about 50%;
- (j) at least about 60%;
- (k) at least about 70%;
- (1) at least about 80%;
- (m) at least about 90%; and
- (n) at least about 95%.

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59. The method of claim 29, wherein said conversion of 2,3-dihydroxyisovalerate to α ketoisovalerate compared to a control host cell containing at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity is increased in an amount selected from the group consisting of:

- (a) at least about 5%;
- (b) at least about 10%;
- (c) at least about 15%;
- (d) at least about 20%;
- (e) at least about 25%;
- (f) at least about 30%;
- (g) at least about 35%;
- (h) at least about 40%;
- (i) at least about 45%;
- (j) at least about 50%;
- (k) at least about 60%;
- (1) at least about 70%;
- (m) at least about 80%;
- (n) at least about 90%; and
- (o) at least about 95%.

60. The recombinant host cell of any one of claims 1, 2, or 5-26, wherein said polypeptide affecting Fe-S cluster biosynthesis is encoded by ARN1, ARN2, ATX1, CCC2, COT1, ENB1, FET3, FET5, FIT1, FIT2, FIT3, FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FTH1, FTR1, HMX1, SIT1, SMF3, TIS11,VHT1, AFT1, AFT2, AIM1, ARH1, ATM1, BUD32, CAD1, CCC1, CFD1, CIA1, CMK1, CTH1, CTI6, CYC8, DAP1, DRE2, ERV1, ESA1, FET4, FRA1, FRA2, GEF1, GGC1, GRX1, GRX2, GRX4, GRX5, HDA1, IBA57, ISA1, ISA2, ISU1, ISU2, JAC1, MGE1, MRS3, MRS4, MSN5, NAR1, NFS1, NFU1, NHP6a, NHP6b, PSE1, SMF1, SNF1, SNF2, SNF3, SNF4, SSQ1, TIM12, TUP1, NP_011911.1, VPS41, YAP5, YFH1, YRA1, ZPR1,

iscAnif, nifU, nifS, cysE1, cysE2, iscS, iscU, iscA, hscB, hscA, Fdx, sufS, sufE, cysE3, sufS2, iscA2, Nfu, nfuA, nfuV, nfu, sufA, sufB, sufC, sufD, sufE1, sufS2, or sufE2

61. A method of measuring the concentration of forms of polypeptide having dihydroxy-acid dehydratase activity, comprising:

(a) separating fractions containing said polypeptide from a crude extract using a first chromatographic procedure;

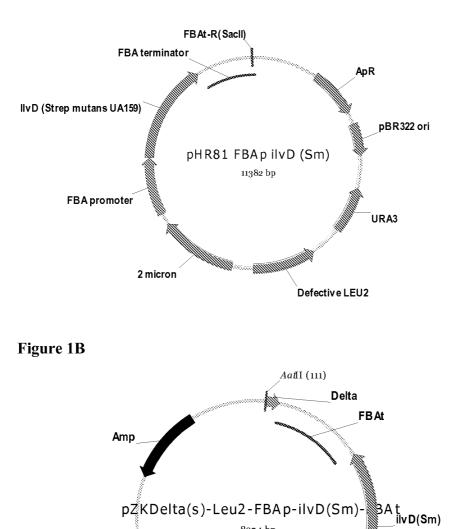
(b) separating fractions containing a form of said polypeptide from (a) using a second chromatographic procedure; and

(c) measuring the concentration of said forms of said polypeptide obtained in (b).

Figure 1A

AscI (5763) Delta

Defective LEU2

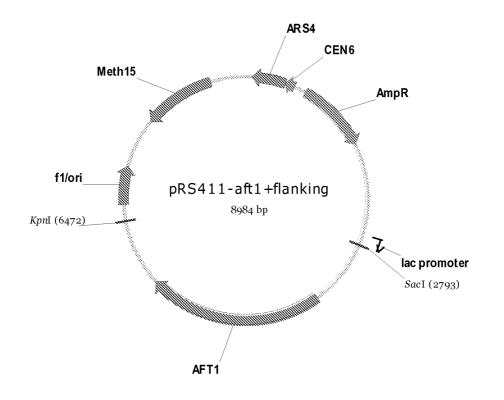


8354 bp

FBAp

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Figure 2





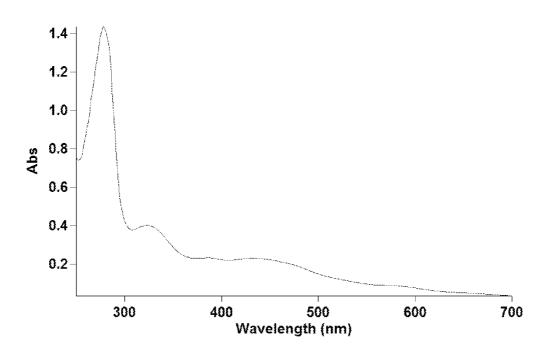
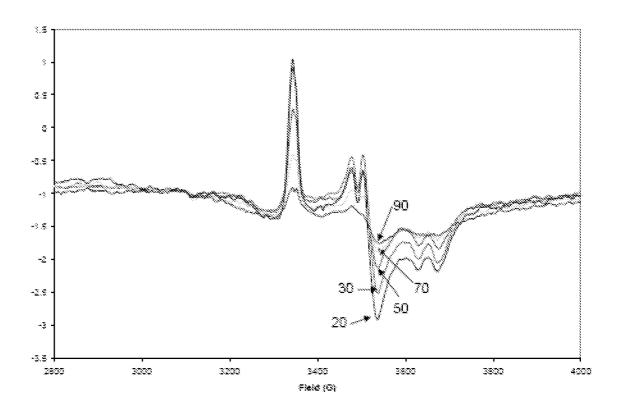
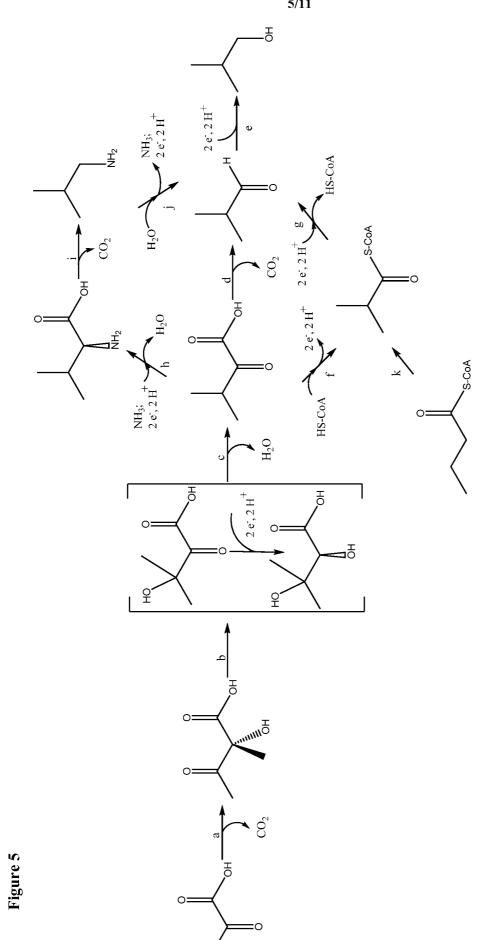


Figure 4





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Figure 6A



Figure 6B

"iscA2"	"nfu"	"mfuA"	"nfuV"

Figure 6C

apo-ISU + 2 IscS-Cys-S-S⁻ + 2 \mathbf{Fe}^{2*} + 2 NFU^{red}

 \rightarrow [2Fe-2S]-ISU + 2 IscS-Cys-S⁺ + 2 NFU^{ox}

Figure 7

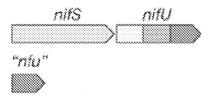
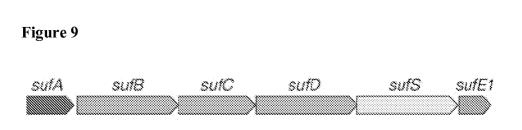
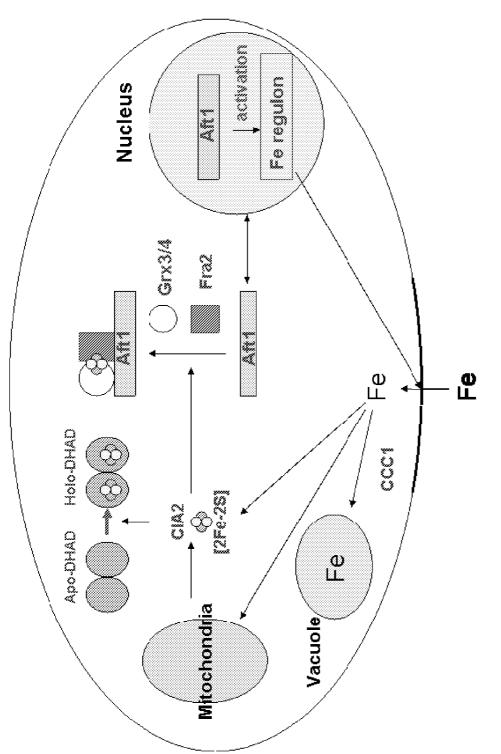


Figure 8

iscR	iseS	iscU k		hscA	fdx orf3





10/11

11/11



