Abstract:

Title: CLONING AND EXPRESSION OF THE GENES ENCODING KEY CLOSTRIDIAL CATALYZING MECHANISMS FOR SYNGAS TO ETHANOL PRODUCTION AND FUNCTIONAL CHARACTERIZATION THEREOF

Sequence listing part of description (Rule 52(a))

The promoter regions of the two operons and the acetyl coA reductase are manipulated to increase ethanol production.
CLONING AND EXPRESSION OF THE GENES ENCODING KEY CLOSTRIDIAL CATALYZING MECHANISMS FOR SYNGAS TO ETHANOL PRODUCTION AND FUNCTIONAL CHARACTERIZATION THEREOF

RELATED U.S. APPLICATION DATA

This application claims the benefit of and priority to United States Patent Application 12/336,238 filed Dec. 16, 2008 as a continuation-in-part application. The entirety of that application is incorporated by reference herein.

FIELD OF THE INVENTION

[0001] This invention relates to the cloning and expression of novel genetic sequences of microorganisms used in the biological conversion of CO, H₂, and mixtures comprising CO and/or H₂ to biofuel products.

BACKGROUND

[0002] Synthetic gas (syngas) is a mixture of carbon monoxide (CO) gas, carbon dioxide (CO₂) gas, and hydrogen (H₂) gas, and other volatile gases such as CH₄, N₂, NH₃, H₂S and other trace gases. Syngas is produced by gasification of various organic materials including biomass, organic waste, coal, petroleum, plastics, or other carbon containing materials, or reformed natural gas.

[0003] Acetogenic Clostridia microorganisms grown in an atmosphere containing syngas are capable of absorbing the syngas components CO, CO₂, and H₂ and producing aliphatic C₂-C₆ alcohols and aliphatic C₂-C₆ organic acids. These syngas components activate Wood-Ljungdahl metabolic pathway 100, shown in FIG. 1, which leads to the formation of acetyl coenzyme A 102, a key intermediate in the pathway. The enzymes
activating Wood-Ljundahl pathway 100 are carbon monoxide dehydrogenase (CODH) 104 and hydrogenase (H₂ase) 106. These enzymes capture the electrons from the CO and H₂ in the syngas and transfer them to ferredoxin 108, an iron-sulfur (FeS) electron carrier protein. Ferredoxin 108 is the main electron carrier in Wood-Ljungdahl pathway 100 in acetogenic Clostridia, primarily because the redox potential during syngas fermentation is very low (usually between -400 and -500 mV). Upon electron transfer, ferredoxin 108 changes its electronic state from Fe³⁺ to Fe²⁺. Ferredoxin-bound electrons are then transferred to cofactors NAD⁺ 110 and NADP⁺ 112 through the activity of ferredoxin oxidoreductases 114 (FORs). The reduced nucleotide cofactors (NAD⁺ and NADP⁺) are used for the generation of intermediate compounds in Wood-Ljungdahl pathway 100 leading to acetyl-CoA 102 formation.

[0004] Acetyl-CoA 102 formation through Wood-Ljungdahl pathway 100 is shown in greater detail in FIG. 2. Either C₀₂ 202 or CO 208 provide substrates for the pathway. The carbon from C₀₂ 202 is reduced to a methyl group through successive reductions first to formate, by formate dehydrogenase (FDH) enzyme 204, and then is further reduced to methyl tetrahydrofolate intermediate 206. The carbon from CO 208 is reduced to carbonyl group 210 by carbon monoxide dehydrogenase (CODH) 104 through a second branch of the pathway. The two carbon moieties are then condensed to acetyl-CoA 102 through the action of acetyl-CoA synthase (ACS) 212, which is part of a carbon monoxide dehydrogenase (CODH/ACS) complex. Acetyl-CoA 102 is the central metabolite in the production of C₂-C₆ alcohols and acids in acetogenic Clostridia.
Ethanol production from Acetyl CoA is achieved via one of two possible paths. Aldehyde dehydrogenase facilitates the production of acetaldehyde, which is then reduced to ethanol by the action of primary alcohol dehydrogenases. In the alternative, in homoacetogenic microorganisms, an NADPH-dependent acetyl CoA reductase ("AR") facilitates the production of ethanol directly from acetyl CoA.

Wood-Ljungdahl pathway is neutral with respect to ATP production when acetate is produced (FIG. 2). When ethanol is produced, one ATP is consumed in a step involving the reduction of methylene tetrahydrafolate to methyl tetrahydrofolate by a reductase, and the process is therefore net negative by one ATP. The pathway is balanced when acetyl-P is converted to acetate.

Acetogenic Clostridia organisms generate cellular energy by ion gradient-driven phosphorylation. When grown in a CO atmosphere, a transmembrane electrical potential is generated and used to synthesize ATP from ADP. Enzymes mediating the process include hydrogenase, NADH dehydrogenases, carbon monoxide dehydrogenase, and methylene tetrahydrofolate reductase. Membrane carriers that have been shown to be likely involved in the ATP generation steps include quinone, menaquinone, and cytochromes.

The acetogenic Clostridia produce a mixture of C₂-C₆ alcohols and acids, such as ethanol, n-butanol, hexanol, acetic acid, and butyric acid, that are of commercial interest through Wood-Ljungdahl pathway. For example, acetate and ethanol are produced by C. ragsdalei in variable proportions depending in part on fermentation conditions. However, the cost of producing the desired product, an alcohol such as ethanol, for example, can be lowered significantly if the production is maximized by
reducing or eliminating production of the corresponding acid, in this example acetate. It is therefore desirable to metabolically engineer acetogenic Clostridia for improved production of selected C$_2$-C$_6$ alcohols or acids through Wood-Ljungdahl pathway by modulating enzymatic activities of key enzymes in the pathway.

**SUMMARY OF THE INVENTION**

[0009] One aspect of the present invention provides novel sequences for three key operons which code for enzymes that catalyze the syngas to ethanol metabolic process: one coding for a carbon monoxide dehydrogenase, a membrane-associated electron transfer protein, a ferredoxin oxidoreductase, and a promoter; a second operon coding for an acetate kinase, phosphotransacetylase, and a promoter, and a third operon coding for an acetyl CoA reductase and a promoter.

[0010] Another aspect of the invention provides an isolated vector or transformant containing the polynucleotide sequence coding for the operons described above.

[0011] Another aspect of the invention provides a method of producing ethanol comprising: isolating and purifying anaerobic, ethanologenic microorganisms carrying the polynucleotides coding for an operon comprising carbon monoxide dehydrogenase, a membrane-associated electron transfer protein, a ferredoxin oxidoreductase, and a promoter; an operon coding for an acetate kinase, phosphotransacetylase, and a promoter, or an operon coding for an acetyl CoA reductase and a promoter; fermenting syngas with said microorganisms in a fermentation bioreactor; providing sufficient growth conditions for cellular production of NADPH, including but not limited to sufficient zinc, to facilitate ethanol production from acetyl CoA.
Another aspect of the invention provides a method of producing ethanol by isolating and purifying anaerobic, ethanologenic microorganisms carrying the polynucleotide coding for acetyl coenzyme A reductase; fermenting syngas with said microorganisms in a fermentation bioreactor; and providing sufficient growth conditions for cellular production of NADPH, including but not limited to sufficient zinc, to facilitate ethanol production from acetyl CoA.

Yet another aspect of the present invention provides a method of increasing ethanologenesis or the ethanol to acetate production ratio in a microorganism containing the nucleotide sequence(s) coding for one of more of the operons described above, said method comprising: modifying, duplicating, or downregulating a promoter region of said nucleotide sequence to increase the activity of the Acetyl Coenzyme A reductase of Claim 16, or to cause overexpression or underexpression of the nucleotide sequence.

The present invention is illustrated by the accompanying figures portraying various embodiments and the detailed description given below. The figures should not be taken to limit the invention to the specific embodiments, but are for explanation and understanding. The detailed description and figures are merely illustrative of the invention rather than limiting, the scope of the invention being defined by the appended claims and equivalents thereof. The drawings are not to scale. The foregoing aspects and other attendant advantages of the present invention will become more readily appreciated by the detailed description taken in conjunction with the accompanying figures.
BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIG. 1 is a diagram illustrating the electron flow pathway during syngas fermentation in acetogenic Clostridia including some of the key enzymes involved in the process;

[0016] FIG. 2 is a diagram illustrating the Wood-Ljungdahl (Ci) pathway for acetyl-CoA production and the enzymatic conversion of acetyl-CoA to acetate and ethanol;

[0017] FIG. 3 is a diagram illustrating a genetic map containing the location of one of the carbon monoxide dehydrogenase (CODH) operons which includes cooS, cooF and a ferredoxin oxidoreductase (FOR), in accordance with the invention;

[0018] FIG. 4 is a diagram illustrating the Wood-Ljungdahl pathway for ethanol synthesis and showing a strategy for specifically attenuating or eliminating acetate production in acetogenic Clostridia by knocking out the genes encoding acetate kinase (ack) and phosphotransacetylase (pta) or by modulating acetate production by mutating or replacing the promoter driving phosphotransacetylase and acetate kinase gene expression, in accordance with the invention;

[0019] FIG. 5 is a diagram of the Wood-Ljungdahl pathway for ethanol synthesis, and shows a strategy for specifically increasing ethanol production in C. ragsdahlei by overexpression of an acetyl CoA reductase in a host knocked out for acetate kinase or phosphotransacetylase activity, in accordance with the invention;

[0020] FIG. 6 is a diagram demonstrating the results of the functional assay wherein the spectrophotometric activity of the gene encoding the acetyl CoA reductase was measured after being ligated into the EcoRI site to form pCOS54.
DETAILED DESCRIPTION

[0021] The present invention is directed to novel genetic sequences coding for acetogenic Clostridia micro-organisms that produce ethanol and acids from syngas comprising CO, CO₂, H₂, or mixtures thereof.

[0022] Several species of acetogenic Clostridia that produce C₂-C₆ alcohols and acids via the Wood-Ljungdahl pathway have been characterized: C. ragsdahlei, C. ljungdahlii, C. carboxydivorans, and C. autoethanogenum. The genomes of three of these micro-organisms were sequenced in order to locate and modify the portions of the genome that code for the enzymes of interest.

[0023] The genes that code for enzymes in the Wood-Ljungdahl metabolic pathway and ethanol synthesis identified in the C. ragsdahlei genome are presented in Table 1. The first column identifies the pathway associated with each gene. The gene identification numbers indicated in the second column correspond to the numbers representing the enzymes involved in the metabolic reactions in the Wood-Ljungdahl pathway shown in FIG. 1 and FIG. 2.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>EC number</th>
<th>ORF ID</th>
<th>Copy ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood-Ljungdahl</td>
<td>1</td>
<td>Carbon Monoxide Dehydrogenase</td>
<td>1.2.2.4</td>
<td>RCCC00183</td>
<td>CODH_1</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>RCCC01175</td>
<td>CODH_2</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>RCCC01176</td>
<td>CODH_3</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>RCCC02026</td>
<td>CODH_4</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td>RCCC03874</td>
<td>CODH_5</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Carbon Monoxide Dehydrogenase / Acetyl-CoA Synthase</td>
<td>1.2.99.2</td>
<td>RCCC03862</td>
<td>cooS/acsA</td>
<td>bifunctional CODH/ACS enzyme, carbon fixation</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Formate Dehydrogenase</td>
<td>1.2.1.2</td>
<td>RCCC00874</td>
<td>FDH_1</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td>RCCC03324</td>
<td>FDH_2</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Formyltetrahydrofolate Synthase</td>
<td>6.3.4.3</td>
<td>RCCC03872</td>
<td>FTHFS</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Methenyltetrahydrofolate cyclohydrolase</td>
<td>3.5.4.9</td>
<td>RCCC03870</td>
<td>MEC</td>
<td>Methyl branch carbon fixation</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Methylenetetrahydrofolate dehydrogenase</td>
<td>1.5.1.5</td>
<td>RCCC03870</td>
<td>MED</td>
<td>Methyl branch carbon fixation</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Methylenetetrahydrofolate reductase</td>
<td>1.5.1.20</td>
<td>RCCC03868</td>
<td>MER</td>
<td>Methyl branch carbon fixation</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Methyltransferase</td>
<td>2.1.1.13</td>
<td>RCCC03863</td>
<td>acsE</td>
<td>Methyl branch carbon fixation</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Corrinoid/Iron-sulfur protein</td>
<td>1.2.99.2</td>
<td>RCCC03864</td>
<td>acsC</td>
<td>Part of CODH/ACS complex, Large subunit</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Corrinoid/Iron-sulfur protein</td>
<td>1.2.99.2</td>
<td>RCCC03865</td>
<td>acsD</td>
<td>Part of CODH/ACS complex, Small subunit</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Acetate Kinase</td>
<td>2.7.2.1</td>
<td>RCCC01717</td>
<td>ACK</td>
<td>Acetate production</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Phospho-transacylase</td>
<td>2.3.1.8</td>
<td>RCCC01718</td>
<td>PTA</td>
<td>Acetate production</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Tungsten-containing aldehyde ferredoxin oxidoreductase</td>
<td>1.2.7.5</td>
<td>RCCC00020</td>
<td>AOR_1</td>
<td>Reduction of acetate to acetaldehyde</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Tungsten-containing aldehyde ferredoxin oxidoreductase</td>
<td>1.2.7.5</td>
<td>RCCC00030</td>
<td>AOR_2</td>
<td>Reduction of acetate to acetaldehyde</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Tungsten-containing aldehyde ferredoxin oxidoreductase</td>
<td>1.2.7.5</td>
<td>RCCC01183</td>
<td>AOR_3</td>
<td>Reduction of acetate to acetaldehyde</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Acetyl-CoA Reductase</td>
<td>1.1.1.2</td>
<td>RCCC02715</td>
<td>ADH_1</td>
<td>zinc-containing, NADPH-dependent Acetyl-CoA reductase</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Alcohol Dehydrogenase</td>
<td>1.1.1.1</td>
<td>RCCC01356</td>
<td>ADH_2</td>
<td>two pfam domain: FeADH and ALDH, AdhE</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Alcohol Dehydrogenase</td>
<td>1.1.1.1</td>
<td>RCCC01357</td>
<td>ADH_3</td>
<td>two pfam domain: FeADH and ALDH, AdhE</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Alcohol Dehydrogenase</td>
<td>1.1.1.1</td>
<td>RCCC01358</td>
<td>ADH_4</td>
<td>two pfam domain: FeADH and ALDH, AdhE, fragment (76aa)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCC03300</td>
<td>ADH_5</td>
<td>one pfam domain: FeADH</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCC03712</td>
<td>ADH_6</td>
<td>one pfam domain: FeADH</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCC04095</td>
<td>ADH_7</td>
<td>one pfam domain: FeADH</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>1.-.-.-</td>
<td>RCCC00004</td>
<td>ADH_8</td>
<td>short chain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ADH, multiple copy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>1.---</td>
<td>RCCC01567</td>
<td>ADH_9 short chain ADH, multiple copy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.---</td>
<td>RCCC02765</td>
<td>ADH_10 short chain ADH, multiple copy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>1.---</td>
<td>RCCC02240</td>
<td>ADH_11 short chain ADH, multiple copy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>1.2.1.10</td>
<td>RCCC03290</td>
<td>ALDH_1 Acetylating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>1.2.1.10</td>
<td>RCCC04101</td>
<td>ALDH_2 Acetylating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>1.2.1.10</td>
<td>RCCC04114</td>
<td>ALDH_3 Acetylating</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>1.12.7.2</td>
<td>RCCC00038</td>
</tr>
<tr>
<td>36</td>
<td>1.12.7.2</td>
<td>RCCC00882</td>
</tr>
<tr>
<td>37</td>
<td>1.12.7.2</td>
<td>RCCC01252</td>
</tr>
<tr>
<td>38</td>
<td>1.12.7.2</td>
<td>RCCC01504</td>
</tr>
<tr>
<td>39</td>
<td>1.12.7.2</td>
<td>RCCC02997</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>RCCC00086</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>RCCC00301</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>RCCC00336</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>RCCC01168</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>RCCC01415</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>RCCC01825</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>RCCC02435</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>RCCC02890</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>RCCC03063</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>RCCC03726</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>RCCC04003</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>RCCC04147</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>Electron carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>RCCC02615</td>
<td>glutamate synthase small chain, but no large chain next to it</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>RCCC02028</td>
<td>next to cooF and cooS, probably important for reduced pyridine cofactor generation</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>RCCC03071</td>
<td>NADH dehydrogenase,</td>
<td></td>
</tr>
</tbody>
</table>
Sequence analysis of the C. ljungdahlii genome was conducted. Genes coding for enzymes in the Wood-Ljungdahl pathway, ethanol and acetate production, and electron transfer have been identified and located within the genome. The results are presented in Table 2.

Table 2. Clostridium ljungdahlii genes used in metabolic engineering experiments.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>EC number</th>
<th>ORF ID</th>
<th>Copy ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood-Ljungdahl</td>
<td>1</td>
<td>Carbon Monoxide Dehydrogenase</td>
<td>1.2.2.4</td>
<td>RCCD00983</td>
<td>CODH_1</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>RCCD00984</td>
<td>CODH_2</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>RCCD01489</td>
<td>CODH_3</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>RCCD04299</td>
<td>CODH_4</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Carbon Monoxide Dehydrogenase / Acetyl-CoA Synthase</td>
<td>1.2.99.2</td>
<td>RCCD00972</td>
<td>CODH_ACS</td>
<td>bifunctional CODH/ACS enzyme, carbon fixation</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Formate Dehydrogenase</td>
<td>1.2.1.2</td>
<td>RCCD01275</td>
<td>FDH_1</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td>RCCD01472</td>
<td>FDH_2</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Formyltetrahydrofolate Synthase</td>
<td>6.3.4.3</td>
<td>RCCD00982</td>
<td>FTHFS</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Methenyltetrahydrofolate cytochrome oxidase</td>
<td>3.5.4.9</td>
<td>RCCD00980</td>
<td>MEC</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Methenyltetrahydrofolate dehydrogenase</td>
<td>1.5.1.5</td>
<td>RCCD00980</td>
<td>MED</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Methenyltetrahydrofolate reductase</td>
<td>1.5.1.20</td>
<td>RCCD00978</td>
<td>MER</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Methyltransferase</td>
<td>2.1.1.13</td>
<td>RCCD00973</td>
<td>MET</td>
<td>Methyl branch carbon fixation</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Corrinoid/Iron-sulfur protein</td>
<td>1.2.99.2</td>
<td>RCCD00974</td>
<td>COPL</td>
<td>Part of CODH/ACS complex, Large subunit</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Corrinoid/Iron-sulfur protein</td>
<td>1.2.99.2</td>
<td>RCCD00975</td>
<td>COPS</td>
<td>Part of CODH/ACS complex, Small subunit</td>
</tr>
<tr>
<td>Ethanol and acetate production</td>
<td>15</td>
<td>Acetate Kinase</td>
<td>2.7.2.1</td>
<td>RCCD02720</td>
<td>ACK</td>
<td>Acetate production</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Phospho-transacetylase</td>
<td>2.3.1.8</td>
<td>RCCD02719</td>
<td>PTA</td>
<td>Acetate production</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Tungsten-containing aldehyde ferredoxin oxidoreductase</td>
<td>1.2.7.5</td>
<td>RCCD01679</td>
<td>AOR_1</td>
<td>Reduction of acetate to acetaldehyde</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>1.2.7.5</td>
<td>RCCD01692</td>
<td>AOR_2</td>
<td>Reduction of acetate to acetaldehyde</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Acetyl-CoA Reductase</td>
<td>1.1.1.2</td>
<td>RCCD00257</td>
<td>ADH_1</td>
<td>zinc-containing, NADPH-dependent Acetyl-CoA Reductase</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCD00167</td>
<td>ADH_2</td>
<td>two pfam domain: FeADH and ALDH, AdhE</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCD00168</td>
<td>ADH_3</td>
<td>two pfam domain: FeADH and ALDH, AdhE</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCD02628</td>
<td>ADH_5</td>
<td>one pfam domain: FeADH</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>1.1.1.1</td>
<td>RCCD03350</td>
<td>ADH_7</td>
<td>one pfam domain: FeADH</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Alcohol Dehydrogenase</td>
<td>1.2.1.10</td>
<td>RCCD00470</td>
<td>ADH_8</td>
<td>short chain ADH, multiple copy</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>1.2.1.10</td>
<td>RCCD01665</td>
<td>ADH_9</td>
<td>short chain ADH, multiple copy</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>1.2.1.10</td>
<td>RCCD01767</td>
<td>ADH_10</td>
<td>short chain ADH, multiple copy</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td>1.2.1.10</td>
<td>RCCD02864</td>
<td>ADH_11</td>
<td>short chain ADH, multiple copy</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Aldehyde Dehydrogenase</td>
<td>1.2.1.10</td>
<td>RCCD02636</td>
<td>ALDH_1</td>
<td>Acetylating</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>1.2.1.10</td>
<td>RCCD03356</td>
<td>ALDH_2</td>
<td>Acetylating</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>1.2.1.10</td>
<td>RCCD03368</td>
<td>ALDH_3</td>
<td>Acetylating</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Hydrogenase</td>
<td>1.12.7.2</td>
<td>RCCD00346</td>
<td>HYD_1</td>
<td>Ni-Fe large subunit, H2 oxidation</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>1.12.7.2</td>
<td>RCCD00938</td>
<td>HYD_2</td>
<td>Ni-Fe small subunit, H2 oxidation</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Hydrogenase</td>
<td>1.12.7.2</td>
<td>RCCD01283</td>
<td>HYD_3</td>
<td>Fe only, large subunit, H2 production</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td></td>
<td>1.12.7.2</td>
<td>RCCD01700</td>
<td>HYD_4</td>
<td>Fe only, H2 production</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>1.12.7.2</td>
<td>RCCD02918</td>
<td>HYD_5</td>
<td>Fe only, H2 production</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>1.12.7.2</td>
<td>RCCD04233</td>
<td>HYD_6</td>
<td>Fe only, H2 production</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>Electron</td>
<td></td>
<td>RCCD00424</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Similarly, the genome of *C. carboxydivorans* was sequenced, and genes coding for the enzymes in the Wood-Ljungdahl pathway and ethanol and acetate synthesis were identified and located. The results are presented in Table 3.

### Table 3. *Clostridium carboxidivorans* genes used in metabolic engineering.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>EC number</th>
<th>ORF ID</th>
<th>Copy ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood-Ljungdahl</td>
<td>1</td>
<td>Carbon Monoxide Dehydrogenase</td>
<td>1.2.2.4</td>
<td>RCCB04039</td>
<td>CODH_1</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>RCCB01154</td>
<td>CODH_2</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>RCCB02478</td>
<td>CODH_3</td>
<td>CO oxidation</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Accession Numbers</td>
<td>Protein Name</td>
<td>Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------------</td>
<td>----------------</td>
<td>-----------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Carbon Monoxide Dehydrogenase / Acetyl-CoA Synthase</td>
<td>RCCB03963, RCCB04038</td>
<td>CODH_4, CODH_5</td>
<td>CO oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCCB04293, CODH_ACS</td>
<td></td>
<td>CO oxidation, bifunctional CODH/ACS enzyme, carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Formate Dehydrogenase</td>
<td>RCCB05406, RCCB01346</td>
<td>FDH_1, FDH_2</td>
<td>Methyl branch carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Formyltetrahydrofolate Synthase</td>
<td>6.3.4.3 RCCB04040</td>
<td>FTHFS</td>
<td>Methyl branch carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Methenyltetrahydrofolate cyclohydrolase</td>
<td>3.5.4.9 RCCB04042</td>
<td>MEC</td>
<td>Methyl branch carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Methylenetetrahydrofolate dehydrogenase</td>
<td>1.5.1.5 RCCB04042</td>
<td>MEC</td>
<td>Methyl branch carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Methylenetetrahydrofolate reductase</td>
<td>1.5.1.20 RCCB04044</td>
<td>MER</td>
<td>Methyl branch carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Methyltransferase</td>
<td>2.1.1.13 RCCB04294</td>
<td>MET</td>
<td>Methyl branch carbon fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Corrinoid/Iron-sulfur protein</td>
<td>1.2.99.2 RCCB04049</td>
<td>COPL</td>
<td>Part of CODH/ACS complex, Large subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Corrinoid/Iron-sulfur protein</td>
<td>1.2.99.2 RCCB04047</td>
<td>COPS</td>
<td>Part of CODH/ACS complex, Small subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Acetate Kinase</td>
<td>2.7.2.1 RCCB05249</td>
<td>ACK</td>
<td>Acetate production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Phospho-transacetylase</td>
<td>2.3.1.8 RCCB02481</td>
<td>PTA</td>
<td>Acetate production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Tungsten-containing aldehyde ferredoxin oxidoreductase</td>
<td>1.2.7.5 RCCB00063</td>
<td>AOR_1</td>
<td>Reduction of acetate to acetaldehyde</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.1.2 RCCB03584</td>
<td>ADH_1</td>
<td>two pfam domain: FeADH and ALDH, AdhE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.1.1 RCCB03870</td>
<td>ADH_2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Alcohol Dehydrogenase</td>
<td>1.1.1.1 RCCB05675</td>
<td>ADH_3</td>
<td>truncated, AdhE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>1.1.1.1 RCCB00958</td>
<td>ADH_5</td>
<td>one pfam domain: FeADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>1.1.1.1 RCCB04489</td>
<td>ADH_6</td>
<td>one pfam domain: FeADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>1.1.1.1 RCCB04503</td>
<td>ADH_7</td>
<td>one pfam domain: FeADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>1.1.1.1 RCCB02465</td>
<td>ADH_9</td>
<td>short chain ADH,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Ethanol and acetate production**
<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>1...</td>
<td>RCCB05551</td>
<td>ADH_10</td>
<td>multiple copy short chain ADH, multiple copy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>1.2.1.10</td>
<td>RCCB02403</td>
<td>ALDH_1</td>
<td>Acetylating</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Aldehyde Dehydrogenase</td>
<td>1.2.1.10</td>
<td>RCCB02561</td>
<td>ALDH_2</td>
<td>Acetylating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>1.2.1.10</td>
<td>RCCB04031</td>
<td>ALDH_3</td>
<td>Acetylating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.12.7.2</td>
<td>RCCB02249</td>
<td>HYD_1</td>
<td>Ni-Fe large subunit, H2 oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td></td>
<td>1.12.7.2</td>
<td>RCCB01319</td>
<td>HYD_2</td>
<td>Fe only, H2 production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td></td>
<td></td>
<td>1.12.7.2</td>
<td>RCCB01405</td>
<td>HYD_3</td>
<td>Fe only, H2 production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td>1.12.7.2</td>
<td>RCCB01516</td>
<td>HYD_4</td>
<td>Fe only, large subunit, H2 production</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td>1.12.7.2</td>
<td>RCCB03483</td>
<td>HYD_5</td>
<td>Fe only, H2 production</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.12.7.2</td>
<td>RCCB05411</td>
<td>HYD_6</td>
<td>Fe only, large subunit, H2 production</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>RCCB00234</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>RCCB00345</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>RCCB01260</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td></td>
<td>RCCB01334</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>RCCB01775</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td></td>
<td>RCCB01960</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>RCCB01972</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td></td>
<td>RCCB02618</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td></td>
<td>RCCB02638</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td></td>
<td>RCCB02836</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>RCCB02853</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>RCCB03023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>RCCB03191</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td></td>
<td>RCCB03278</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>RCCB03452</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td></td>
<td>RCCB03596</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td></td>
<td>RCCB03762</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>RCCB03972</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td></td>
<td>RCCB04165</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td></td>
<td>RCCB04383</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td></td>
<td>RCCB04571</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td></td>
<td>RCCB04585</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td></td>
<td>RCCB05780</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td></td>
<td>RCCB05975</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Electron carrier**

**Ferredoxin**
Genes that code for enzymes in the electron transfer pathway include carbon monoxide dehydrogenase, Enzyme Commission number (EC 1.2.2.4). Five separate open reading frame (ORF) sequences were identified in C. ragsdalei and C. ljungdahlii, and six were identified in the C. carboxidivorans genome for the carbon monoxide dehydrogenase enzyme.

FIG. 3 is a diagram of carbon-monoxide dehydrogenase operon 300. The gene order within operon 300 is highly conserved in all three species of acetogenic Clostridia, and comprises the genes coding for the carbon monoxide dehydrogenase (cooS) (Gene ID
4, Tables 1, 2, and 3), followed by the membrane-associated electron transfer FeS protein (cooF) (Gene ID 55, Table 1; Gene ID 51, Table 2; Gene ID 67, Table 3), in turn, followed by ferredoxin oxidoreductase (FOR).

[0028] A comparison was conducted of the genetic sequence found in the operon of FIG. 3 across the three species of acetogenic Clostridia. The cooS gene had 98% identity between c. ragsdalei and c. ljungdahlii, 84% identity between c. carboxydovorans and c. ragsdalei, and 85% identity between c. carboxydovorans and c. ljungdahlii. The cooF gene had 98% identity between c. ragsdalei and c. ljungdahlii, 80% identity between c. carboxydovorans and c. ragsdalei, and 81% identity between c. carboxydovorans and c. ljungdahlii. The FOR gene had 97% identity between c. ragsdalei and c. ljungdahlii, 77% identity between c. carboxydovorans and c. ragsdalei, and 77% identity between c. carboxydovorans and c. ljungdahlii.

[0029] Six hydrogenase (EC 1.12.7.2) ORF sequences were identified in the genome of each of the acetogenic Clostridium species.

[0030] Twelve ferredoxin biosynthesis genes (Gene ID 40-51) were identified in the C. ragsdalei genome. Eleven ferredoxin biosynthesis genes (Gene ID 37-47, Table 2) were found in C. ljungdahlii, and twenty-six (Gene ID 36-61, Table 3) were found in C. carboxydovorans.

[0031] Three genes coding for ferredoxin oxidoreductase enzymes were found in the C. ragsdalei genome that contain both a ferredoxin and nicotinamide cofactor binding domain. The ORF Sequence ID numbers (Table 1) for these genes are: RCCC02615; RCCC02028; and RCCC03071. The key gene for metabolic engineering, RCCC02028, is part of the cooS/cooF operon, also shown in FIG. 3. Similarly, three genes coding for
ferredoxin oxidoreductase (FOR) enzymes were found in the C. ljungdahlii genome. Each of these genes code for both the ferredoxin and cofactor binding domains. The ORF Sequence ID numbers for these genes are: RCCD00185; RCCD01847; and RCCD00433 (Table 2). The key gene RCCD01847, is part of the cooF/cooS operon shown in FIG. 3.

[0032] Five genes were found in the C. carboxidivorans genome that contain both the ferredoxin and cofactor binding domains. The ORF Sequence ID numbers (Table 3) for these genes are: RCCB00442; RCCB01674; RCCB03510; RCCB00586; and RCCB04795. The potentially key gene for modulating electron flow is RCCB03510, which is part of the cooF/cooS operon (FIG. 3).

[0033] The genes encoding AR (Gene ID 21, Table 1; Gene ID 19, Table 2) were sequenced in C. ragsdalei and C. ljungdahlii. A high degree of gene conservation is observed for the acetyl CoA reductase gene in C. ragsdalei and C. ljungdahlii. Furthermore, in both micro-organisms, the enzyme exhibits a high degree of homology. The sequence of the acetyl CoA gene in c. ragsdalei and c. ljungdahlii was compared and found to have a 97.82% identity.

[0034] Further, the functionality of the gene (including the promoter) encoding for acetyl CoA reductase was tested. The gene was amplified by PCR, transferred into shuttle vector pCOS52 and ligated into the EcoRI site to form pCOS54. The vector contained the entire acetyl-CoA reductase gene and its promoter on a high-copy plasmid. pCOS52 contained the same backbone vector as pCOS54 but lacked the AR gene. pCOS52 was used as the control plasmid in functional assays to determine expression of
the AR gene in E. coli to confirm the Clostridial gene function. The results are shown in FIG. 7, and confirm the function of the acetyl CoA reductase gene.

[0035] The functional assay consisted of adding cells harvested at the given time points to a reaction buffer containing NADPH and acetone as the substrate. Spectrophotometric activity (conversion of NADPH to NADP+) was measured at 378nm and compared to a standard curve to determine total activity level. Specific activity was determined using 317 mg/gram of dry cell weight at an OD measurement of 1.

[0036] The genes encoding the PTA-ACK operon (Gene IDs 16-17, Tables 1 and 3; Gene IDs 15-16, Table 2) and its promoter were sequenced in C. ragsdaisi, C. ljungdahlii, and C. carboxydivorans. The functionality of the operon was confirmed, and it was demonstrated that downregulation of the operon increases the ethanol to acetate production ratio. Downregulation involves decreasing the expression of the transcription of the 2-gene operon via promoter modification through site-directed mutagenesis. Such downregulation leads to a decrease in mRNA, leading to a decrease in protein production and a corresponding decrease in the ability of the strain to produce acetate. Such downregulation can be achieved via the method described in Example 2.

[0037] Additionally, a comparison was conducted of the genetic sequence found in the PTA-ACK operon across three species of acetogenic Clostridia. The PTA gene had 97% identity between c. ragsdaisi and c. ljungdahlii, 78% identity between c. carboxydivorans and c. ragsdaisi, and 79% identity between c. ljungdahlii and c. carboxydivorans. The ACK gene had 96% identity between c. ragsdaisi and c. ljungdahlii, 78% between c. carboxydivorans and c. ragsdaisi, and 77% between c. carboxydivorans and c. ljungdahlii.
[0038] Key genes to promote production of ethanol in C. ragsdahlei include:

SEQ ID NO 1 (Gene ID Nos. 4, 55, 53, Table 1) the gene sequence, including the experimentally determined promoter region, for carbon monoxide dehydrogenase, coos, electron transfer protein cooF, and the NADH dependent ferredoxin oxidoreductase (FOR);

SEQ ID NO 2 (Gene ID Nos. 17, 16, Table 1), the gene sequence, including the experimentally determined promoter region, for ACK and PTA;

SEQ ID NO 3 (Gene ID No. 6, Table 1), the gene sequence, including the experimentally determined promoter region, for the acetyl CoA reductase;

Sequence Listing

C. ragsdalei gene sequences (Table 1)

>SEQ ID NO. 1: (cooS, cooF, NADFLFerredoxin Oxidoreductase operon (includes STOP), Gene ID Nos. 4, 55, 53)

```
TATTATATCAATATAAATTTTTCAATCAATATAAAGAATTATTTATATTT
ATATTGACAGAAGAAGCAAGTTTTTATTTATATTATTTATTTTTATATT
ATTTTTTTAGGTTAGTTGTACTTTGAAATAAATAATGATAACTTAAATATA
CTATTACAGTTTTTGATTCTTAGATAAGATATCTTATCTTTAGCAGTTTT
GATACGTGTTACATGAGGAATAATCCTAATCAGTAATTTTAATAATTTA
AGTATAACTTAATAGTATAAGTTGGAGTTTTTTATTATGCTAAATAACAAAAT
ATGTAAGTCAGCAGATAAAGGTAACTTTGAAAGTTTTATAGTCTTCTAGATG
AGAAACTTCTCCTCATAAGGTGAAAGCAAGAGTTAAATGTGTTTTTGTT
CAGCTAGGATCTGCCTGCTAGACTCTGTGCAACAGGCTCCCTGCAAGATAAC
CTAAAAGCTCCAAGAGGAGTATGTGTTCTACTGCTAGATACATGGTTGCAAG
AAACTTTCTTAGACTGCTAGCTGCGGCAGTGAGTATTATACATGCTAGAC
AAAATACAGCTAGAAAGCTAAATACGTAGTTGAAACCGCGGAGAGATAAA
AAGGAATGAAATGCTCTCAACACCTAGCAAAAAACTTGTATAAAGAATAC
TGAACCCACATAAAAAAGCTGTAGCTAGCTGTGCAACAGGCTCCCTGCAAGATAAC
AAAAAACAAAAATTCGAAAAAATGGGAACTTATATAAAATAGCTTATGCTAC
TAGATCCTAGAAAATAAATTAATATAAATCTGAGCTGCGGAGAAAAAACT
GAAGGTTTATGAGTTGTAGTTAAACCCTCCTCAAATCTCAGCAGAAGACCT
GATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
TGAGATGAGTTTGTTAAATTTGCTGTTAAATTGCTCTGCTAGTTATTAC
AGGATATGCTCTAAATTTGTTAAAAACTTGGGAAATACCTACAGTGGATTTAAC
TGTTACCCCTAACAAATTTTATATGACATATTTAGGTGAAACCTGCTAAGAC
ACTAGTTTTCCTAAGATGCTGTTTGGTCTAGTTACCATGCGGTAACTTACT
```
CCTGAAGCTGTAAAAAAAGCCCAAGCAGTTGGTGCTAAAGGATTCAAACTAGTTGGATGTACCTGTGTCGGACAGGATTTACAGTTAAGAGGTAAATACTATACTGATGTTTTCTCCGTCATGCAGGAAATAACTTTACAAGTGGAAGCCTAATAGCAACTGGAAGTTGACATGCAATAGTATCTGAATTTAACTGTA...
ATATGGCCTATAGCTGAAAGCAGGGAATAACTGCTGCTTACAACATGGTAG
GTATAAATAGAGAATTTATCAGACAATCTTTTGGCATGAAGAATATAGCTGAAAGTT
ATATGAATTTTTATTTTGGCATGACACTTTTGGCATGACATTCTCTACTGCGGCG
TACATGGATATCTCATAAAAAAAATATTAAAGCAATATCCTATAAAAAAAATATTTCG
AGATGGAAGTAGTTACCGGTGCACTTCTAAGGTGAGATATATCTCATTACAAA
ATTATTTTGACATAGATTATTCTCATTCTAGTTGGAAGGAGAGATGGAACATAAT
ATAGTTACAAATTGCGTCTAAAC

SEQ ID NO. 2: (PTA-ACK operon (includes STOP), Gene ID Nos. 17, 16)
GCATACTGATGTATTTTTATTTTATTTGGAAATGCTGAAATATAGATGATCATTAAACATGGTAG
ATATGAATTTTTATTTTGGCATGACACTTTTGGCATGACATTCTCTACTGCGGCG
TACATGGATATCTCATAAAAAAAATATTAAAGCAATATCCTATAAAAAAAATATTTCG
AGATGGAAGTAGTTACCGGTGCACTTCTAAGGTGAGATATATCTCATTACAAA
ATTATTTTGACATAGATTATTCTCATTCTAGTTGGAAGGAGAGATGGAACATAAT
ATAGTTACAAATTGCGTCTAAAC
Using detailed genomic information, the acetogenic Clostridia micro-organisms have been metabolically engineered to increase the carbon and electron flux through the biosynthetic pathways for ethanol and butanol, while simultaneously reducing or eliminating carbon and electron flux through the corresponding acetate and butyrate pathways.
formation pathways, in accordance with the present invention. For this purpose, the activities of key genes encoding for enzymes in the pathway have been modulated. In one embodiment, gene expression of key alcohol producing enzymes is increased by increasing the copy number of the gene. For example, a key carbon monoxide dehydrogenase operon (FIG. 3) and the associated electron transfer proteins, including acetyl CoA reductase and aldehyde ferredoxin oxidoreductase are duplicated within the genome of the modified organism. In one embodiment, these duplications are introduced into strains having knocked out or attenuated acetate production to further channel electrons into the ethanol or butanol production pathway. In another embodiment a knockout strategy is applied to strains of acetogenic Clostridia that, when grown on syngas, produce more complex mixtures of alcohols and acids, such as ethanol, butanol and hexanol and their corresponding carboxylic acids.

[0040] In one embodiment, vectors to be used for the transfer of acetogenic Clostridia cloned genes from cloning vehicles to parent acetogenic Clostridia strains are constructed using standard methods (Sambrook et al., 1989). All gene targets used in molecular genetics experiments are amplified using high-fidelity polymerase chain reaction (PCR) techniques using sequence-specific primers. The amplified genes are next subcloned into intermediate cloning vehicles, and later recombined in multi-component ligation reactions to yield the desired recombinant vector to be used in the gene transfer experiments. The vectors contain the appropriate functional features required to carry out the gene transfer experiments successfully and vary depending on the method used.

[0041] To transfer the recombinant vectors into recipient acetogenic Clostridia, a variety of methods are used. These include electroporation, bi-parental or tri-parental
conjugation, liposome-mediated transformation and polyethylene glycol-mediated transformation. Recombinant acetogenic Clostridia are isolated and confirmed through molecular biology techniques based on the acquisition of specific traits gained upon DNA integration.

[0042] Example 1

[0043] Acetogenic Clostridia contain operon 300, shown in FIG. 3, that consists of carbon monoxide dehydrogenase 104 (cooS, Gene ID 4, Table 1, Table 2, Table 3), a membrane-associated electron transfer protein (cooF), and a ferredoxin oxidoreductase (FOR). Overexpression of carbon monoxide dehydrogenase 104 within the acetogenic Clostridia is known to increase electron flow from syngas components to the oxidized nucleotide cofactors NAD+ and NADP+. The increased levels of reduced nucleotide cofactors then stimulate generation of intermediate compounds in Wood-Ljungdahl pathway.

[0044] In one embodiment, operon 300 is amplified using long-PCR techniques with primers that are designed to anneal to a region 200 nucleotides (nt) upstream of the carbon monoxide dehydrogenase gene and 200 nt downstream of the ferredoxin oxidoreductase gene. The total region is about 3.8 kilobase pairs. The amplified DNA is cloned directly into suitable plasmid vectors specifically designed to ligate PCR products such as pGEM T easy (Promega, Madison, WI) or pTOPO (Invitrogen, Carlsbad, CA). The ends of the PCR product contain engineered restriction sites to facilitate later cloning steps. The operon 300 is subcloned into a vector that already contains cloned
chromosomal C. ragsdalei or other acetogenic Clostridial DNA to allow chromosomal integration at a neutral site.

[0045] Example 2

[0046] Because carboxylic acids compete with alcohols for electrons, decreasing acid production allows more electrons to flow down the alcohol-production pathway from the CoA intermediate directly to the alcohol. Acetogenic Clostridia contain genes for phospho-transacetylase enzyme (Gene ID 17, Tables 1 and 3; Gene ID 16, Table 2) that converts acetyl-CoA to acetyl-phosphate and acetate kinase (Gene ID 16, Table 1) that converts acetyl-phosphate 218 to acetate 214. In one embodiment, genetic modifications to delete all or part of the genes for both enzymes and knock out or attenuate production of acetate are made as shown in FIG. 4.

[0047] Using PCR and other standard methods, a recombinant vector containing two large non-contiguous segments of DNA is generated. Upon replacement of the native gene by the recombinant vector gene, the Clostridial strain contains no phosphotransacetylase or acetate kinase activities as shown in FIG 4 by X 504 and X 502, respectively.

[0048] Modulation of the common promoter region, P* 506 to attenuate gene expression of phosphotransacetylase 508 and acetate kinase 510 and subsequent acetate production are carried out by generating a series of recombinant vectors with altered promoter regions. The vector series is constructed by site-directed mutagenesis.

[0049] Additionally, down-regulation of the 2-gene operon containing pta/ack genes is performed by site-directed mutagenesis of the promoter region. A decrease in RNA polymerase binding leads to a decrease in transcriptional activity off of the pta/ack
promoter and in turn lead to a decrease in protein activity. The end result is a decrease in acetate production since the intermediates are produced at a lower rate and more carbon from acetyl-CoA goes towards ethanol production. A promoter probe assay using a reporter group that is easily quantitated has been developed to measure relative promoter strength of the pta/ack promoter in vivo. After site-directed mutagenesis is performed, which imparts single and multiple lesions over a 200 base pair region, strains that have decreased promoter activity are isolated such that a series of strains with 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% and 0% activity of the native promoter in the assay are isolated and tested in recombinant Clostridia strains.

[0050] Example 3

[0051] In vivo, the acetyl CoA enzyme designated in 102 and FIG 4 converts the Coenzyme A (CoA) form of a carbon moiety, such as acetyl-CoA 102 or butyryl-CoA directly to its corresponding alcohol. Thermodynamically, direct conversion from the CoA form to the alcohol requires transfer of four electrons, and is a more efficient way to generate the alcohol, compared to the two-step conversion of the carboxylic acid to the corresponding alcohol. For example, as shown in FIG 5, the two step conversion requires that acetate 214, first be converted to its aldehyde form (acetaldehyde, 604), and then to the corresponding alcohol, ethanol 216. Thus, increasing AR activity, portrayed by the vertical arrow 602 is desirable for increasing alcohol production, and increasing the selectivity of the process by increasing the ratio of alcohol to acid.

[0052] In one embodiment, AR activity in acetogenic Clostridia is increased by amplifying the gene in vitro using high-fidelity PCR and inserting the duplicated copy of
the gene into a neutral site in the chromosome using standard molecular genetic
techniques. After gene replacement of the vector, the chromosome contains two copies
of the AR. Confirmation of genereplacement followed by gene expression studies of the
recombinant strain are performed and compared to the parent strain.

[0053] In other embodiments a similar strategy is used to increase the enzymatic activity
of adhE-type alcohol dehydrogenases, short-chain alcohol-dehydrogenases and primary
Fe-containing alcohol dehydrogenases.

[0054] Example 4

[0055] Under some conditions, Clostridia need to obtain additional energy in the form
of adenosine triphosphate production (ATP) causing the cells to temporarily increase the
production of acetate 214 from acetyl-CoA 102. The net reaction is 1 ATP from ADP +
Pi through acetyl-phosphate. Acetate production is advantageous to the syngas
fermentation process at low to moderate acetic acid concentrations, because it allows the
cells to produce more energy and remain robust. However, too much free acetic acid
causes dissipation of the transmembrane ion gradient used as the primary ATP generation
source and therefore becomes detrimental to the cells. For industrial production purposes,
it is advantageous to convert the acetate to ethanol to increase ethanol production and
reduce the probability of accumulating too much free acetic acid.

[0056] In one embodiment, ethanol production in the double mutant C. ragsdahlei strain
is increased by between 10 and 40% as a result of the increased aldehyde ferredoxin
oxidoreductase and AR activities. In another embodiment, the ratio of ethanol to acetate
produced is increased between 5 and 10 fold, but allows sufficient acetate formation to support ATP production needed to meet the energy needs of the microorganism.

[0057] While the invention has been described with reference to particular embodiments, it will be understood by one skilled in the art that variations and modifications may be made in form and detail without departing from the spirit and scope of the invention.
CLAIMS

What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence encoding an operon that codes for carbon monoxide dehydrogenase, a membrane-associated electron transfer protein, a ferredoxin oxidoreductase, and a promoter, said sequence being at least 77% identical to SEQ ID NO. 1.

2. A vector comprising the polynucleotide of Claim 1.

3. An isolated transformant containing the polynucleotide of Claim 1.

4. An isolated transformant carrying the vector of Claim 2.

5. An antisense nucleic acid to the nucleotide sequence of Claim 1, said antisense nucleic acid inhibiting the expression of the enzyme.

6. A method of producing ethanol comprising: isolating and purifying anaerobic, ethanologenic microorganisms carrying the polynucleotide of Claim 1; fermenting syngas with said microorganisms in a fermentation bioreactor.
7. A method of increasing ethanologenesis in a microorganism containing the nucleotide sequence of Claim 1, said method comprising: modifying or duplicating a promoter region of said nucleotide sequence to increase the activity of the operon of Claim 1 or to cause overexpression of the operon.

8. An isolated polynucleotide comprising a nucleotide sequence encoding an operon that codes for acetate kinase, phosphotransacetylase, and a promoter, said sequence being at least 76% identical to SEQ ID NO 2.


10. An isolated transformant containing the polynucleotide of Claim 8.

11. An isolated transformant carrying the vector of Claim 10.

12. An antisense nucleic acid to the nucleotide sequence of Claim 8, said antisense nucleic acid inhibiting the expression of the enzyme.

13. A method of producing ethanol comprising: isolating and purifying anaerobic, ethanologenic microorganisms carrying the polynucleotide of Claim 8; and fermenting syngas with said microorganisms in a fermentation bioreactor.
14. A method of increasing ethanologenesis in a microorganism containing the nucleotide sequence of Claim 8, said method comprising: modifying or downregulating a promoter region of said nucleotide sequence to decrease the activity of the operon of Claim 8 or to cause underexpression of the operon.

15. An isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide encoding an acetyl coenzyme A reductase and a promoter, said sequence being at least 98% identical to SEQ ID NO. 3.

16. A vector comprising the polynucleotide of Claim 15.

17. An isolated transformant carrying the polynucleotide of Claim 15.

18. An isolated transformant carrying the vector of Claim 16.

19. An antisense nucleic acid to the nucleotide sequence of Claim 15, said antisense nucleic acid inhibiting the expression of the enzyme.

20. A method of producing ethanol comprising: isolating and purifying anaerobic, ethanologenic microorganisms carrying the polynucleotide of Claim 16; fermenting syngas with said microorganisms in a fermentation bioreactor; providing sufficient growth conditions to facilitate the production of ethanol from acetyl CoA via the reductase of Claim 15.
21. A method of increasing ethanologenesis in a microorganism containing the nucleotide sequence of Claim 15, said method comprising: modifying or duplicating a promoter region of said nucleotide sequence to increase the activity of the Acetyl Coenzyme A reductase of Claim 15 or to cause overexpression of the nucleotide sequence.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/53(2006.01)i, C12N 15/63(2006.01)i, C12N 15/113(2010.01)i, C12P 7/06(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/53; C12N 9/90; C12P 7/10; C12N 1/21; C12P 7/06

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

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal), PubMed & Google

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>WO 2008-122354 A1 (JOHANN WOLFGANG GOETHE-UN IVERSTITAT FRANKFURT AM MAIN) 16 October 2008 See abstract and claims 8, 35</td>
<td>15-21</td>
</tr>
<tr>
<td>A</td>
<td>WO 2008-018930 A2 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.) 14 February 2008 See abstract and claim 49</td>
<td>15-21</td>
</tr>
<tr>
<td>FX</td>
<td>KOPKE, H. et al., Clostridium ljungdahlii represents a microbial production platform based on syngas, Proc. Natl. Acad. Sci. USA, 20 Jul. 2010, Vol. 107, No. 29, pp. 13087-13092 See figures 1, 2</td>
<td>15-21</td>
</tr>
</tbody>
</table>

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
18 AUGUST 2011 (18.08.2011)

Date of mailing of the international search report
18 AUGUST 2011 (18.08.2011)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 189 Cheongsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
Park, Jung Mm
Telephone No. 82-42-481-8291

Form PCT/ISA/210 (second sheet) (July 2009)
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

   2. ☑ Claims Nos.: 1-14  
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   
   In the filed sequence listing of electronic form, the sequences according with SEQ ID NO. 1 and 2 are not described. Therefore, a meaningful search on claims 1-14 has not been made.

   3. ☐ Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☑ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☑ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☑ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest  ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DE 102007016534 A1</td>
<td>09.10.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2139998 A1</td>
<td>06.01.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2010-151548 A1</td>
<td>17.06.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2660147 A1</td>
<td>21.02.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 101679939 A</td>
<td>24.03.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2054503 A2</td>
<td>06.05.2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2054503 A4</td>
<td>01.12.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2010-500026 A</td>
<td>07.01.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2010-500026 T</td>
<td>07.01.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MX 200900194 A</td>
<td>15.04.2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2010-112656 A1</td>
<td>06.05.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wo 2008-02 1141 A3</td>
<td>21.02.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2650505 A1</td>
<td>14.02.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 101657541 A</td>
<td>24.02.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2041293 A2</td>
<td>01.04.2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2010-524428 A</td>
<td>22.07.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2010-524428 T</td>
<td>22.07.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TW 200813219 A</td>
<td>16.03.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wo 2008-018930 A3</td>
<td>14.02.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 1999-18586 A1</td>
<td>09.09.1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 1999-47441 A1</td>
<td>04.11.1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 2203495 A</td>
<td>23.10.1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 672748 B2</td>
<td>17.10.1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2097803 A1</td>
<td>08.06.1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2097803 C</td>
<td>03.08.2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2106377 A1</td>
<td>19.09.1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2106377 C</td>
<td>04.11.2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1065915 C</td>
<td>16.05.2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1068629 C</td>
<td>18.07.2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1070424 A</td>
<td>31.03.1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1070424 CO</td>
<td>31.03.1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1091774 A</td>
<td>07.09.1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1091774 CO</td>
<td>07.09.1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1342773 AO</td>
<td>03.04.2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1342773 CO</td>
<td>02.01.2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0431047 A1</td>
<td>12.06.1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0560885 A1</td>
<td>04.06.1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0560885 B1</td>
<td>17.03.1999</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
<td>Publication date</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>EP 0576621 B1</td>
<td>28.02.2001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JP 03-457664 B2</td>
<td>01.08.2003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JP 03-593125 B2</td>
<td>24.11.2004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KR 10-0292079 B1</td>
<td>30.03.2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05000000 A</td>
<td>19.03.1991</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05028539 A</td>
<td>02.07.1991</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05424202 A</td>
<td>13.06.1995</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05482846 A</td>
<td>09.01.1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05487989 A</td>
<td>30.01.1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05554520 A</td>
<td>10.09.1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05821093 A</td>
<td>13.10.1998</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 05916787 A</td>
<td>29.06.1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 06107093 A</td>
<td>22.08.2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 2003-0008363 A1</td>
<td>09.01.2003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 6849434 B2</td>
<td>01.02.2005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 7192772 B1</td>
<td>20.03.2007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wo 90-02-193 A1</td>
<td>08.03.1990</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wo 92-10561 A1</td>
<td>25.06.1992</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wo 92-16615 A1</td>
<td>01.10.1992</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wo 94-06924 A1</td>
<td>31.03.1994</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>