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VIEWPOINT

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Extremely Thermophilic Routes to Microbial Electrofuels

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INTRODUCTION

Economic and political instability, as well as rising concerns about the environmental impacts of excess atmospheric carbon dioxide, have prompted research into production of liquid transportation fuels from renewable sources.[1,2] As such, renewable fuels produced by microbial hosts have leapt forward in the past five years because of increasing sophistication in genetic manipulation of key intermediary pathways, metabolic engineering, and directed evolution. Advanced microbial biofuel production has been achieved, with examples ranging from short chain alcohols,[3−9] to products derived from isoprenoids[10−12] and fatty acids.[13−15] As an example, high levels of butanol production by a recombinant *Escherichia coli* strain was recently demonstrated using biosynthetic pathways from *Clostridia*, providing proof of the robustness of metabolically engineered microorganisms and their potential use for large-scale fuel production.[16]

As is the case for current fermentation-based biofuel production such as ethanol and isopropanol, microbial routes require a reduced form of carbon for host strain growth and maintenance, typically in the form of sugars, peptides, or plant oil.[4,17] As such, these approaches inherit some of the basic challenges of photosynthetic, plant-based liquid fuel production. They are still hampered by the low efficiency of converting sunlight into usable chemical energy, they still utilize food crops for feedstock and thus compete with human and animal consumption, driving food prices upward, and they still require large areas of land to capture enough solar energy for large scale production of liquid fuels.[18,19] However, the next trend emerging in the quest for optimizing microbial fuel generation seeks to avoid these problems by directly creating a usable liquid fuel without wasteful intermediary steps.

Biofuel production currently relies on the "fuel" (i.e., carbohydrates) created by plants to provide the reducing power needed to sustain microorganisms, as they create a fuel more compatible with internal combustion engines. In the new paradigm (Figure 1), the so-called "electrofuels" are created by harnessing the energy of low-potential electrons—which could be delivered by inorganic substrates such as hydrogen, reduced metals, or even electric current—to drive the direct fixation of carbon dioxide by microbes using molecular machinery borrowed from one of nature's carbon fixation pathways. Once the inorganic carbon has been reduced and incorporated into the microbial host cell's central metabolism, it can then be transformed into the desired fuel or organic product.

Depending upon the target system, the low-potential electrons can come from a variety of sources. There are many well-studied examples of autotrophic microorganisms that can utilize a broad range of inorganic substrates as sources of energy, including H_2, H_2S, S, CO, NH_3, metal sulfides, or reduced metal ions.[20,21] Some organisms, such as those within the genera *Geobacter* and *Shewanella*, can grow by accepting electrons directly from a cathode.[22] Recent studies have shown that certain acetogenic organisms can produce acetate and small amounts of other organic compounds from carbon dioxide when grown in biofilms on graphite electrodes.[23,24] Hydrogen gas, which is used by many microorganisms for reducing power, is a promising energy carrier that could bridge conventional and renewable energy generation strategies to biologically driven fuel production systems.

There are also a variety of known pathways for fixing (reducing) carbon dioxide to a usable carbon form. In addition to the Calvin−Benson−Bassham cycle, the ubiquitous biochemical pathway for carbon fixation found in plants and cyanobacteria, there are at least five other distinct pathways that microorganisms use to grow autotrophically, as shown in Table 1.[21] Like the Calvin cycle, the reductive citric acid cycle is widely distributed, while the reductive acetyl-CoA pathway (Wood−Ljungdahl) is found only in organisms growing close to the thermodynamic limit, such as methanogens and acetogens. The C-3 hydroxypropionate cycle has so far only been detected in *Chloroflexus*, a green nonsulfur thermophile. The two most recently discovered pathways for carbon fixation, the C-4 hydroxybutyrate cycles, are unique to thermophilic and hyperthermophilic archaea (T_{opt} 70−100 °C). The explosion in genomic sequence and metagenomic data in the past decade has been instrumental to the discovery of these new C-3 and C-4 pathways, which operate in both aerobic and anaerobic archaea.[20,25] The pathways from high temperature organisms possess unique advantages common to all thermophilic enzymes and, as such, could be exploited advantageously in a genetically designed microbial fuel production host. The enzymes are active and stable at high temperatures, which can yield significant bioprocessing advantages for large-scale production of liquid fuels. Moreover, a production system designed for a hyperthermophilic host growing at 90 °C could enable continuous product recovery, thereby providing an avenue for process intensification and minimizing solvent toxicity issues at high production levels.

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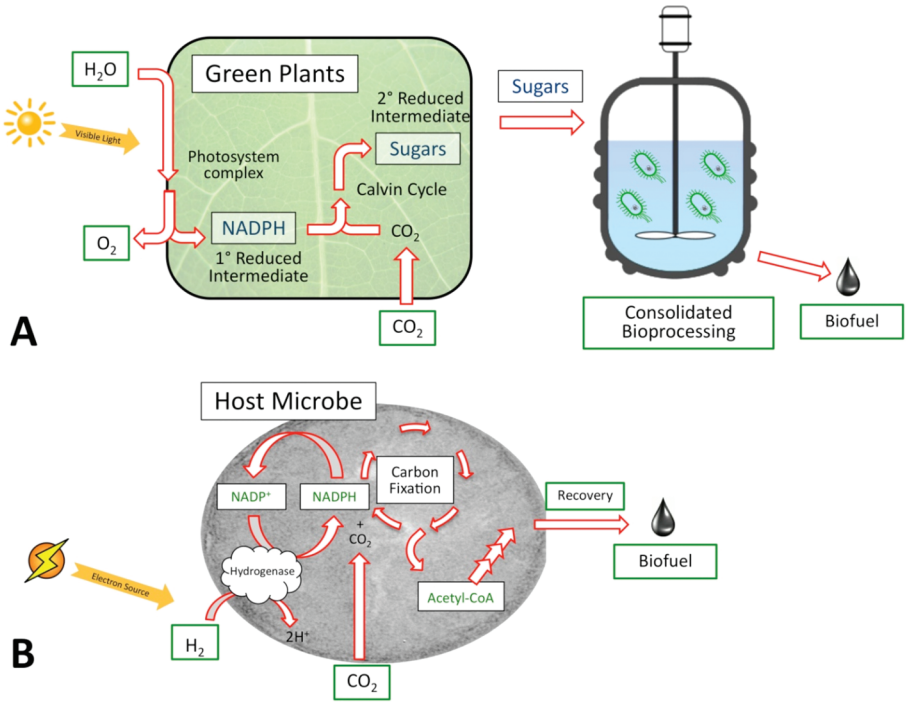

Figure 1. Alternative routes to biofuels. (A) In conventional biofuels, water donates electrons that are excited by sunlight via the photosystem complex. The reducing power drives carbon fixation via the Calvin cycle to make a reduced carbon compound, i.e., sugar. The sugar is then fermented by microorganisms to produce the desired fuel molecule. (B) In the electrofuels paradigm, the microbial host utilizes low-potential electrons to generate reducing power, which is used to drive production of the desired fuel or organic compound.

Table 1. — Pathways for Carbon Fixation

pathway	organisms	reductants	key enzyme(s)
reductive pentose phosphate[26]	plants, algae, cyanobacteria, aerobic proteobacteria (α-, β-, γ-types), Purple bacteria[a]	NAD(P)H	rubisCO, phosphoribulokinase
reductive citric acid cycle[27]	green sulfur bacteria, Proteobacteria (δ-, ε- types) *Aquificae, Nitrospirae*	NAD(P)H and ferredoxin	2-oxoglutarate synthase, ATP-citrate lyase
reductive acetyl-CoA pathway[28,29]	acetogenic bacteria, methanogenic archaea, planctomycetes, sulfate-reducing bacteria, *Archaeoglobales*	ferredoxin	acetyl-CoA synthase-CO dehydrogenase
hydroxypropionate bicycle[30,31]	*Chloroflexaceae*	NAD(P)H	malonyl-CoA reductase, propionyl-CoA synthase, malyl-CoA lyase
hydroxypropionate/hydroxybutyrate cycle[32]	(micro)aerobic *Sulfolobales*	NAD(P)H	acetyl-CoA-propionyl-CoA carboxylase, 4-hydroxybutyryl-CoA dehydratase
dicarboxylate/hydroxybutyrate cycle[33]	anaerobic *Thermoproteales, Desulfurococcales*	NAD(P)H and ferredoxin	4-hydroxybutyryl-CoA dehydratase

[a] Purple bacteria use the Calvin cycle as an electron sink for anaerobic photoheterotrophic growth.[34]

Until recently, the use of metabolic pathways from hyperthermophiles, which grow optimally above 80 °C, was limited by the availability of acceptable genetic hosts. Most genetic systems were developed for mesophilic microorganisms, whose optimal growth temperatures are well below the lower threshold for hyperthermophilic enzyme activity. The development of a genetic system in a hyperthermophilic host, one in which the selected pathways could operate at or near their temperature optimum, has been an elusive target until recently. Developing a genetic system for any hyperthermophilic organism is complicated by the instability of common antibiotics or resistance proteins at high temperatures, as well as differences in the molecular machinery of archaea compared to bacteria.[35,36] Groundbreaking work by Sato et al.[37,38] and Santangelo et al.[39,40] overcame these challenges to create a robust transformation system for *Thermococcus kodakaraensis* (T_{opt} 85 °C).[41] Around the same time, work done on hyperthermophilic *Sulfolobus* spp. (T_{opt} 80 °C) provided methods for gene disruption, shuttle vectors, and inducible expression of recombinant tagged protein.[42−45] These same techniques have also been successfully applied to a naturally

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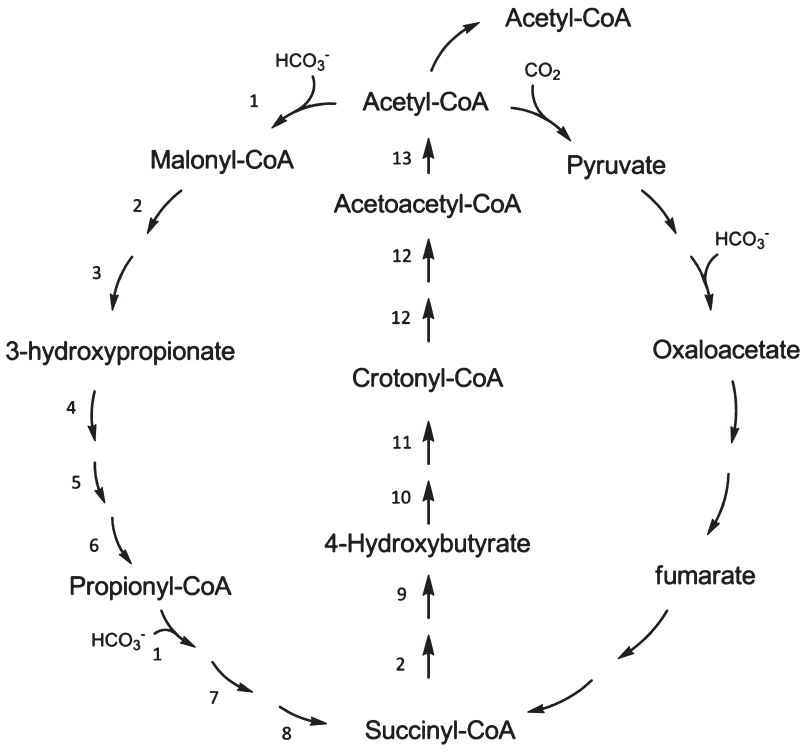

Figure 2. Reaction schema for the 3-hydroxypropionate/4-hydroxybutyrate (left side) and dicarboxylate/4-hydroxybutyrate (right side) carbon fixation cycles. Numbers indicate the unique enzymes in the 3-HP/4-HB pathway: 1, acetyl-CoA/propionyl-CoA carboxylase; 2, malonyl-CoA/succinyl-CoA reductase; 3, malonic semialdehyde reductase; 4, 3-hydroxypropionate-CoA ligase; 5, 3-hydroxypropionyl-CoA dehydratase; 6, acryloyl-CoA reductase; 7, methylmalonyl-CoA epimerase; 8, methylmalonyl-CoA mutase; 9, succinic semialdehyde reductase; 10, 4-hydroxybutyrate-CoA ligase; 11, 4-hydroxybutyryl-CoA dehydratase; 12, crotonyl-CoA/(*S*)-3-hydroxybutyryl-CoA dehydrogenase; 13, acetoacetyl-CoA β-ketothiolase.

competent strain of *Pyrococcus furiosus*, a marine anaerobe that grows optimally near 100 °C.[36] These new hyperthermophilic host strains are exciting developments in the field and hold promise as novel platforms for high temperature in vivo fuel production.

THERMOPHILIC CARBON FIXATION

Autotrophic carbon fixation is the essential starting point for all organic biochemical synthesis. Assimilating carbon dioxide (+4) into cellular carbon building blocks (average oxidation state of 0) requires four reducing equivalents and an input of energy via ATP hydrolysis. The first known carbon fixation pathway was the photosynthetic, reductive pentose phosphate pathway, discovered in the 1940s in Melvin Calvin's laboratory.[26] Found in plants, algae, cyanobacteria, and many aerobic proteobacteria, the enzymes of this robust pathway are stable in the presence of oxygen. It was thought to be the sole pathway for carbon fixation until the reductive citric acid cycle was discovered in *Chlorobium thiosulfatophilum* in the 1960s.[27] The reductive acetyl-CoA pathway (Wood−Ljungdahl), described fully in the mid-1980s, is used by acetogens and methanogens for both carbon fixation and energy conservation via generation of an electrochemical gradient.[28,29]

In 1989 a new pathway for autotrophic fixation was discovered in the thermophilic green nonsulfur bacterium *Chloroflexus aurantiacus*.[30,46] In this pathway, now referred to as the 3-hydroxypropionate (3-HP) bicycle, acetyl-CoA (C-2) is carboxylated to (*S*)-malyl-CoA (C-3) via succinyl-CoA. (*S*)-malyl-CoA is then cleaved to produce glyoxylate and regenerate acetyl-CoA. However, since glyoxylate is not a central metabolite, the process requires a second cycle to assimilate glyoxylate and produce pyruvate.[31,47] The pathway has been found in a few other members within the family Chloroflexaceae, but is not widely distributed and seems to have evolved independently.[21]

While the final details of the hydroxypropionate bicycle were being substantiated, pioneering work by Fuchs and co-workers led to the discovery of two carbon fixation pathways in archaea that were based on the *C. aurantiacus* 3-HP bicycle.[32,33] Using the growing database of genomic sequence data, they searched for organisms containing acetyl-CoA carboxylase genes similar to the one found in *Chloroflexus*. These were found in several organisms in the Crenarchaeota, including *Metallosphaera* and *Sulfolobus* spp., as well as in the Euryarchaeota, such as *Archaeoglobus* sp. These organisms all possessed the enzymes for the first half of the 3-HP pathway but lacked the complete cycle.

Although these archaea come from different habitats, they all possess a common and unique gene encoding for 4-hydroxybutyryl-CoA dehydratase, a [4Fe-4S] cluster and flavin adenine dinucleotide-containing enzyme.[32] This enzyme was known to play a role in 4-aminobutyrate fermentation in *Clostridia*, but previously had only been found in strictly anaerobic bacteria.[48] It catalyzes a radical-mediated dehydration by abstracting the least activated hydrogen atom in the C3 position of the butyryl chain, accomplishing what would be a very difficult abiotic chemical transformation.[49] In this case, the enzyme appeared to be involved in a novel pathway for regenerating acetyl-CoA by reducing succinyl-CoA to 4-hydroxybutyrate (4-HB). Now believed to be composed of 16 reactions catalyzed by 13 enzymes, the hydroxypropionate/hydroxybutyrate (3-HP/4-HB) pathway uses NADPH as reductant and requires four molecules of ATP

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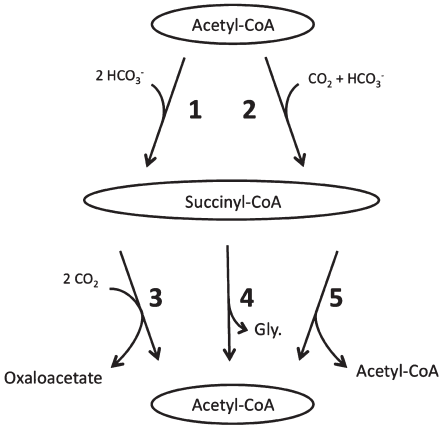
for each molecule of acetyl-CoA produced from two carbon dioxide molecules (Figure 2).[21]

The same gene for 4-hydroxybutyrl-CoA dehydratase was also found in species of the anaerobic and hyperthermophilic *Ignicoccus*, but these archaea lacked the genes required for the first part of the pathway found in the 3-HP bicycle. Instead, these organisms use two different carboxylating enzymes, pyruvate synthase and phoshoenolpyruvate carboxylase, to make succinyl-CoA via pyruvate and oxaloacetate (Figure 3).[50] The route from succinyl-CoA back to acetyl-CoA in the anaerobic dicarboxylate/hydroxybutyrate pathway is the same as in the aerobic Crenarchaeota, suggesting a common evolutionary ancestor. The enzymes in this pathway preferentially use reduced ferredoxin instead of NAD(P)H as the electron donor, and the pathway requires only three ATP molecules for each molecule of acetyl-CoA produced.[33]



Key Intermediates
1 – Oxaloacetate
2 – 3-Hydroxypropionate
3 – Citrate
4 – Malyl-CoA
5 – 4-Hydroxybutyrate

Pathways
1 + 3 = Reductive citric acid cycle
2 + 4 = 1st cycle in 3-hydroxypropionate bicycle (Gly = Glyoxylate)
1 + 5 = 3-hydroxypropionate / 4-hydroxybutyrate cycle
2 + 5 = dicarboxylate / 4-hydroxybutyrate cycle

Figure 3. Common metabolic strategies found in microbial carbon fixations pathways. Adapted with permission from Huber et al.[33] Copyright 2008 Proceedings of the National Academy of Sciences U.S.A. (PNAS).

Most of the enzymes of this new 3-HP/4-HB carbon fixation pathway have since been biochemically confirmed by Fuchs and co-workers.[51−56] Our transcriptomic studies[57] of *M. sedula* comparing heterotrophic and autotrophic growth modes confirmed several genes that were suspected to be involved in carbon fixation based on annotation or homology to other known autotrophic pathways (Figure 4). For enzymes having several possible gene candidates, such as 4-hydroxybutryl-CoA dehydratase (E11) or acetoacetyl-CoA β-ketothiolase (E13), the transcriptomic analysis gave additional supporting information to identify the most likely gene-encoding open reading frame. To date neither of these two enzymes have been successfully produced in a recombinant host, although the clostridial version of 4-hydroxybutyryl-CoA dehydratase has been purified and studied in detail.[48,49,58] Two of the other previously unknown genes, encoding for enzymes that convert crotonyl-CoA into acetoacetyl-CoA, were recently discovered to be encoded by a single bifunctional fusion enzyme.[56] Currently, there has been little work done to recombinantly produce or biochemically characterize the enzymes from *I. hospitalis*. Their properties and potential advantages/disadvantages in any bioprocessing system are therefore unknown.

The carbon fixation routes based on 3-HP and 4HB could provide the basis for creating not just fuels, but many other small molecule organic compounds from basic metabolites such as acetyl-CoA. In fact, it may be that these thermophilic pathways, in view of their simple and efficient design, are related to the earliest forms of CO_2 fixation developed by microorganisms, well before photosynthesis emerged on earth. That same simplicity and efficiency are now attractive features for a genetically engineered, electrofuel-producing microorganism.

UTILIZATION OF HYDROGEN GAS AS AN ENERGY CARRIER

Hydrogen is a simple molecule of growing interest for its value as an energy carrier. In the automotive industry, it has been employed in powering vehicles via internal combustion or fuel cell technology.[2] Hydrogen can be generated via steam reformation of natural gas, partial oxidation of hydrocarbons, microbial

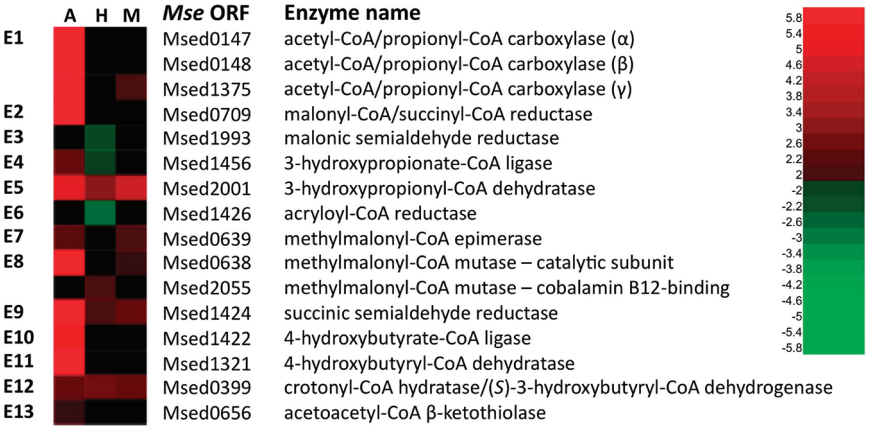


Figure 4. Transcriptomic patterns in 3-Hydroxypropionate/4-Hydroxybutyrate carbon fixation cycle in *Metallosphaera sedula* under (A)utotrophic, (H)eterotrophic, and (M)ixotrophic conditions, updated from Auernik and Kelly.[57] Note up-regulation of putative pathway enzymes for growth under autotrophic conditions. Red signifies high transcription and green signifies low transcription. Results from Mixed Effects ANOVA model yielding least-squares means of normalized $\log_2$ transformed transcription levels, relative to the overall average transcription level of 0.

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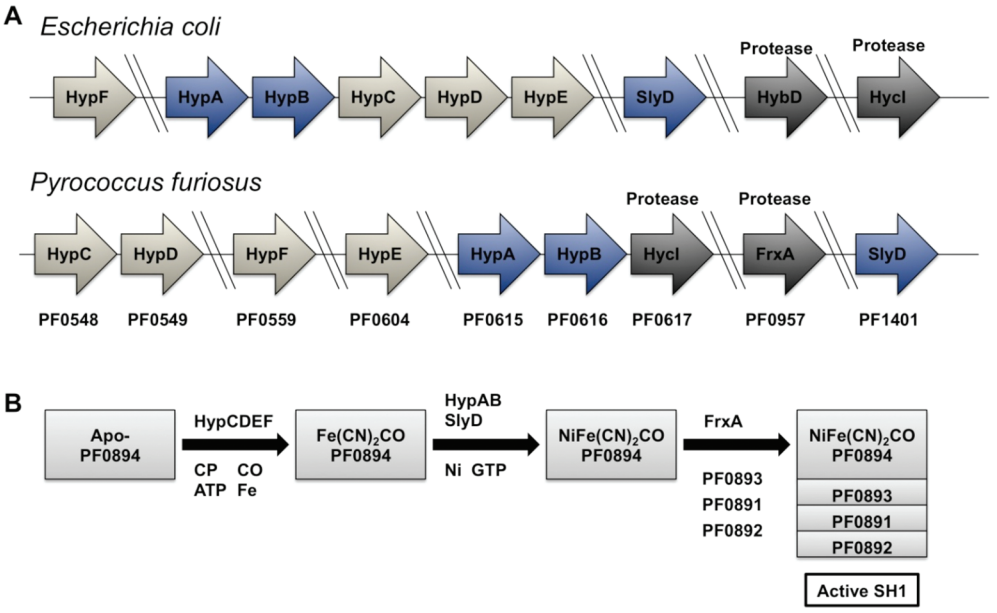

Figure 5. (A) Homologues of the eight types of hydrogenase assembly genes encoded within the genomes of *E. coli* and *P. furiosus*. (B) Assembly process for *P. furiosus* soluble hydrogenase 1 (SHI). This begins with insertion of $Fe(CN)_2CO$ into the catalytic subunit, which is encoded by PF0894. CP represents carbamoyl phosphate, the source of the cyanide ligand, while CO represents the source of the CO, the donor of which is not known. In the second step the nickel atom is inserted to give the inactive subunit. Maturation to give the fully functional heterotetrameric enzyme requires C-terminal processing by FrxA and the binding of the three additional subunits, encoded by PF0891, PF0892, and PF0893.

fermentation of biomass, or electrolysis using electricity from conventional or renewable sources.[59−61]

Hydrogenases are enzymes capable of catalyzing the generation or oxidation of gaseous hydrogen. Microorganisms that contain such enzymes can evolve hydrogen from biomass degradation or utilize hydrogen as an energy source for biosynthesis.[62] These enzymes catalyze the reversible conversion of molecular hydrogen (H_2) to protons (H^+) and electrons (e^-). This process is exploited by all three domains of life as a means of capturing energy, disposing of reducing equivalents, generating a proton gradient, acid resistance, or as a part of mixed acid fermentation.[63−66]

The mesophilic bacterium, *E. coli*, contains three different hydrogenases, referred to as Hyd-1, Hyd-2 and Hyd-3. All three are membrane-bound and only synthesized when the organism is grown in the absence of oxygen. The three hydrogenases are of the nickel−iron type, in which catalysis takes place at a binuclear [NiFe] site. In the active site, the two metals are linked by the sulfur atoms of two cysteinyl residues and the iron atom is activated by the coordination of diatomic ligands (two −CN and one −CO).[65] Hyd-1 and Hyd-2 are part of respiratory systems that consume hydrogen to produce low potential electrons while Hyd-3 couples hydrogen production to mixed acid fermentation via formate.[67]

The catalytic subunit containing the [NiFe]-site is highly conserved in the microbial world. For example, a search of the genome of the hyperthermophilic archaeon *P. furiosus*, which grows optimally at 100 °C and is very distantly related to *E. coli*, reveals three [NiFe]-hydrogenases. Two are cytosolic and utilize NADPH as an electron carrier (SHI and SHII), while the third is membrane bound (MBH) and functions to evolve hydrogen while pumping protons to create an electrochemical gradient.[68−70] SHI and SHII are thought to recycle hydrogen produced for biosynthetic purposes.

The biosynthesis of any NiFe-hydrogenase is a complicated process requiring the participation of eight accessory proteins (Figure 5). Despite their phylogenic distance, *P. furiosus* and *E. coli* share homologues of the [NiFe]-hydrogenase assembly machinery. In both organisms, the terminal processing step in producing a functional hydrogenase involves cleavage of a C-terminal peptide from the catalytic subunit by a protease specific for that hydrogenase.[64] The other accessory proteins are encoded by the so-called hyp genes, hypA-F,[71] together with a chaperone protein termed SlyD, which helps in the recruitment of HypB, a nickel-binding GTP-ase.[72] HypC, HypD, HypE, and HypF assemble the cyanide and carbon monoxide ligands at the iron site, which is then inserted into the catalytic subunit of hydrogenase.[71] HypC remains bound to the hydrogenase large subunit while HypA, HypB, and SlyD insert nickel to complete the catalytic [NiFe] site.[72,73] In the case of *E. coli* Hyd-3, the protease HycI is responsible for the cleavage of 20 amino acid residues from the C-terminus of the large subunit to generate the active form of the hydrogenase.[63] In *P. furiosus*, the protease required for the cleavage of the C-terminus of the catalytic subunit of SHI is termed FrxA.[64]

The production of recombinant [NiFe]-hydrogenase in a genetically tractable microorganism is complicated by the complexity of the biosynthetic process required to generate a catalytically active hydrogenase, as well as the oxygen-sensitivity of the enzyme. However the production of *P. furiosus* SHI was recently demonstrated in *E. coli*.[64] Although homologues of the processing genes for *P. furiosus* SH1 are present in *E. coli*, their ability to process recombinant SH1 could not be assumed. Therefore, the strategy used to produce recombinant SH1 in *E. coli* initially was with the total set of thirteen genes required by *P. furiosus*: four genes that encode the four subunits of the enzyme, seven genes that encode the maturation proteins, and two genes that encode proteases (at that time it was not known which of the two *P. furiosus* proteases, FrxA and HycI, were active toward SHI). Since the hydrogenases of both *P. furiosus* and *E. coli* are

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inactivated by oxygen, the expression of the thirteen *P. furiosus* genes was placed under control of the *E. coli hya* promoter, which is induced by anaerobic conditions. The genes were transformed into *E. coli* using four plasmids (each with a different antibiotic resistance marker). To ensure that the only hydrogenase activity that could be measured was that of recombinant SHI, a strain of *E. coli* was used in which the genes encoding the catalytic subunits of its own three hydrogenases, Hyd-1, Hyd-2 and Hyd-3, had been all deleted.

Use of the *hya* promoter allowed the recombinant *E. coli* strain containing the thirteen *P. furiosus* genes to be grown up under aerobic conditions until it reached the appropriate cell density, at which point the expression of all foreign genes was induced simply by replacing the air feed with an inert gas (nitrogen). After a short period the activity of recombinant SHI could be measured by a hydrogenase assay at 80 °C.[64] Using *E. coli* strains lacking one or more of the four plasmids, it was demonstrated that FrxA rather than HycI was specific for processing SH1 and that FrxA could not be replaced by the proteases of *E. coli*. However, all of the other *Pyrococcus* maturation proteins were not essential, as the *E. coli* maturation proteins were sufficient to obtain recombinant SHI. Hence, recombinant SHI could be generated by simply expressing five *P. furiosus* genes in *E. coli:* FrxA and the four genes encoding the four subunits of SHI.[64] Amazingly, this shows that despite the phylogenetic distance between *P. furiosus* and *E. coli*, the assembly machinery for the [NiFe]-hydrogenase is highly conserved.

While the physiological role of SHI in *P. furiosus* has been controversial,[69] kinetic analyses have shown that the rate of hydrogen consumption is an order of magnitude higher than the rate of hydrogen evolution at 80 °C using NADPH as the electron carrier.[70] This ability to capture low potential electrons from hydrogen make SHI a very useful enzyme in electrofuel production since these low potential electrons can directly drive CO_2 fixation and fuel synthesis pathways.

GENETIC MANIPULATION OF *P. FURIOSUS*

To date, no hyperthermophilic microorganism, defined as having optimal growth at 80 °C or above, has been used as a recombinant host for biofuel production. To tap into the newly discovered archaeal thermophilic 3-HP/4-HB CO_2 fixation pathways, one would need a hyperthermophilic host in which genetic manipulation is possible. As noted above, there are two groups of hyperthermophilic archaea for which genetics have already been developed: the *Sulfolobales* and the *Thermococcales*.[74] The *Sulfolobales* are aerobic thermoacidophiles and the first targeted mutant within this group was created using a natural β-galactosidase (*lacS*) mutant of *Sulfolobus solfataricus* (*Ss*, T_{opt} 80 °C), where the α-amylase (*amyA*) coding sequence was disrupted by insertion of a modified allele of the *Ss lacS* gene.[42] Targeted gene disruptions in *Ss* have also been used to establish the role of two mercury resistance gene homologues (*merA* and *merR*) in mercury resistance.[75] More recently, deletion mutants have been obtained in both *S. islandicus* and *S. acidocaldarius*.[76−78]

The *Thermococcales* are obligately anaerobic hyperthermophiles, and genetic techniques were first developed for *Thermococcus kodakarensis* (*Tk*), which grows optimally at 85 °C. It was shown to be naturally competent and to recombine added DNA into its genome.[38,79] Auxotrophic strains for uracil (Δ*pyrF*), tryptophan (Δ*trpE*), and agmatine (Δ*pdaD*) have been developed as background strains for genetic manipulation.[38,79,80] In addition, β-galactosidase has been utilized as a reporter gene.[39] More recently, a naturally competent variant of *P. furiosus* was discovered.[36] Development of a genetic system in *P. furiosus* has been based on that of the closely related *Tk*. A deletion mutant in *P. furiosus* was constructed in a gene (*pyrF*) required for uracil biosynthesis by double crossover homologous recombination. The *pyrF* deletion strain (COM1) is a naturally competent uracil auxotroph that has been successfully used to generate markerless deletion mutants, including those involved in hydrogen metabolism.[36]

An ideal host for heterologous expression of the archaeal thermophilic CO_2 fixation pathway for the production of biofuels would be a genetically amenable hyperthermophile that produces H_2 and captures low potential electrons in the form of NADPH. *P. furiosus* fits all these criteria; it grows between 70 and 103 °C (T_{opt} 100 °C), has a doubling time as low as 37 min, is genetically tractable, produces H_2 as an end product of sugar fermentation, and has the ability to generate NADPH from H_2.[81] This system could, therefore, provide a platform for the (over)expression of thermophilic CO_2 fixation pathway genes (and any desired biofuel biosynthesis genes). By integration into the chromosome under the control of constitutive or inducible native promoters, production of acetyl-CoA and its conversion to desired biofuels can be optimized.

A key feature of the archaeal thermophilic CO_2 fixation pathway is that the high-energy reductant is supplied as NADPH. As discussed above, *P. furiosus* contains three distinct NiFe-hydrogenases, one membrane-bound (MBH) and two soluble (SHI and SHII), that have been purified and characterized in their active states.[68,69,82] A major advantage of using *P. furiosus* as a host for thermophilic CO_2 fixation is the presence of SHI. This enzyme is active over a wide temperature range (30−100 °C), has a relatively high affinity for H_2, and its H_2-uptake activity has been shown to be an order of magnitude higher than its H_2-evolution activity. It is therefore proposed to use H_2 for the regeneration of NADPH.[69] Interestingly, recent single- and double-deletion mutants of the gene cluster encoding SHI did not show any effect on cell growth.[36] Clearly, SHI is not essential for growth under laboratory conditions. This represents a great advantage for using *P. furiosus* as a host for the CO_2 fixing-pathway, since it should be possible to create a recombinant strain containing a high concentration of SHI, without affecting cell viability, that would be even more efficient than the wild type strain in using H_2 to produce NADPH for CO_2 fixation.

Combining expression of a thermophilic 3-HP/4-HB carbon fixation pathway with a host, such as that described above, generates a recombinant system that can be used to demonstrate H_2-dependent CO_2 fixation into key intermediates, 3-HP, 4-HB, and acetyl-CoA. By generating acetyl-CoA, the activated C2 unit can be utilized directly to generate a range of biofuels, including alkanes, biodiesel (fatty acid esters), and ethanol, as well as butanol. There would be no need to add specific metabolites to obtain the key intermediates or the end products (3-HP, 4-HB, acetyl-CoA). Finally, the broad temperature range for growth of hyperthermophilic hosts (between 70 and above 100 °C) means that the source of CO_2 fixation genes used for recombinant expression can come from a range of organisms such as *M. sedula*, which grows optimally near 75 °C, *Ss*, which grows optimally at 85 °C, and even *Ignicoccus* strains growing near 100 °C.

OTHER CONSIDERATIONS

Whatever the organism or carbon fixation mechanism chosen for creating a microbial electrofuel production host, there are a

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few issues of concern in bringing a process to scale that should be considered during the early stages of design. Carbon dioxide availability to the host organism will likely be the limiting factor in fuel production, given the low solubility of gaseous CO_2 at high temperatures. In fact most of the carbon fixation pathways mentioned in this review require bicarbonate instead of CO_2.[21] Overexpression of carbonic anhydrase might be necessary to maximize the rate-limiting carboxylation step and improve overall production.

At large scale, the availability and cost of CO_2 will also be an important factor that determines the overall economic feasibility of this approach. Carbon capture technologies, especially when coupled with emissions reductions programs at existing coal- and natural gas-fired power plants, could operate in tandem with electrofuels production facilities to provide the large quantities of CO_2 needed. Research into carbon capture technologies has been stimulated by international concerns about high global CO_2 emissions and the effects that excess atmospheric CO_2 will have on climate patterns.[83,84] Scaling-up any process that utilizes hydrogen gas as a redox mediator also requires careful design to minimize potential explosion hazards. Using an anaerobic organism, such as *P. furiosus*, minimizes these hazards within the reactor since oxygen is not present.

Lastly, the issue of product recovery is vital to ensure that high production levels do not generate a toxic liability to the host culture. Most organisms can only tolerate between 1 and 2% *n*-butanol (v/v).[85] A great deal of effort has been invested in creating strains that have higher tolerance to the toxic effects of organic solvents, such as butanol.[86−88] Another approach is to design a system so that the solvent can be continuously removed before toxicity effects impact the host. For example, Shen et al. used gas stripping to remove butanol from the system and achieve high production rates.[16] A system using *P. furiosus* as a host could leverage the growth temperature of the organism (T_{opt} 98 °C) to facilitate product recovery. Butanol forms a heteroazeotrope with water at 93 °C, which would facilitate continuous recovery by distillation or decanting liquid directly from the top of the fermentation vessel.

CONCLUSION

The use of microbial hosts as platforms for production of liquid fuels has already been conceptually established and demonstrated.[13,89] In some cases, these optimized systems are producing yields equal to or exceeding traditional fermentation-based production methods.[16] By integrating a carbon fixation module into the host, it may be possible to create liquid fuels directly from carbon dioxide using hydrogen gas as the sole source of energy and reducing power. This process would bypass the use of plant-based feedstock and could potentially be much more efficient than photosynthetic approaches. In addition to being highly scalable for industrial use, the process permits flexibility in energy inputs that could use both conventional energy generation sources as well as intermittent renewable sources such as wind and solar.

Hyperthermophilic enzymes for carbon fixation have not yet been imported or operated inside of a synthetic host. The development of new tools, particularly the development of genetic systems for other hyperthermophilic organisms, may open the doorway to new types of modified hosts. The opportunities here are valuable both for advancing the science and also addressing pressing energy and environmental concerns.

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