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Physiological, Metabolic and Biotechnological Features of Extremely Thermophilic Microorganisms

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Abstract

The current upper thermal limit for life as we know it is approximately 120°C. Microorganisms that grow optimally at temperatures of 75°C and above are usually referred to as ‘extreme thermophiles’ and include both bacteria and archaea. For over a century, there has been great scientific curiosity in the basic tenets that support life in thermal biotopes on earth and potentially on other solar bodies. Extreme thermophiles can be aerobes, anaerobes, autotrophs, heterotrophs, or chemolithotrophs, and are found in diverse environments including shallow marine fissures, deep sea hydrothermal vents, terrestrial hot springs – basically, anywhere there is hot water. Initial efforts to study extreme thermophiles faced challenges with their isolation from difficult to access locales, problems with their cultivation in laboratories, and lack of molecular tools. Fortunately, because of their relatively small genomes, many extreme thermophiles were among the first organisms to be sequenced, thereby opening up the application of systems biology-based methods to probe their unique physiological, metabolic and biotechnological features. The bacterial genera *Caldicellulosiruptor*, *Thermotoga* and *Thermus*, and the archaea belonging to the orders Thermococcales and Sulfolobales, are among the most studied extreme thermophiles to date. The recent emergence of genetic tools for many of these organisms provides the opportunity to move

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beyond basic discovery and manipulation to biotechnologically relevant applications of metabolic engineering.

Graphical Abstract

Thermal springs from Yellowstone National Park, USA

Introduction

Extreme thermophiles are distinct from other organisms due to their ability to subsist optimally at temperatures in excess of 75°C. Their survival in these harsh environments piqued the interest of curious microbiologists as far back as the turn of the 20th century. In fact, one of the earliest reports of thermophiles occurred in 1903 describing bacterial samples taken from pools in Yellowstone National Park[1]. Although this drew interest and debate about the limits of life and our evolutionary history, the study of thermophiles did not begin in earnest until the 1960's. Around this time, extensive sampling projects in Yellowstone lead to the isolation of *Thermus aquaticus*[2] (an aerobic bacterium with optimal growth between 70–75°C), known for its DNA polymerase that revolutionized the field of molecular biology through its use in the Polymerase Chain Reaction (PCR). This enzyme in particular represented one of the earliest uses of thermally-stable enzymes for a biotechnological application. The next few decades yielded the discovery of thermophiles around the globe in extremely diverse environments, ranging from volcanoes and calderas to deep sea smoker vents to terrestrial mud pools[3–7].

The apparent diversity and novelty of these microbes likely drove early research in this field to uncover the molecular machinery central to their survival. In fact, some of the earliest sequenced genomes were extremophiles[8–10], furthering efforts to understand the molecular and genetic basis for thermophily and the evolution of life. However, a lack of genetics tools has impeded the extensive study of these organisms by traditional approaches (i.e., gene deletions to understand the consequences of loss of function). In lieu of more traditional methods, the availability of genomic data for many extreme thermophiles supported 'omics'-based approaches to ascertain the function of specific genes and their roles in the unique biochemistry of these organisms. As such, the merger of systems biology (e.g., transcriptomics and genomics), traditional microbiological studies, and newly emerging genetic systems[11] are opening the door for metabolic engineering opportunities to bring extreme thermophiles into the technological limelight. This will allow for these organisms to be utilized as sources of uniquely functioning enzymes, optimized niche industrial strains, and novel metabolic engineering platforms. Such opportunities for biotechnological application are already being pursued for members of the bacterial genera

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Caldicellulosiruptor and *Thermotoga* and for archaea belonging to the orders Thermococcales and Sulfolobales. Here we present a brief overview of these extremely thermophilic organisms, with the intention of highlighting potential biotechnological applications, which exploit their distinctive metabolisms.

Sulfolobales

Perhaps the most distinctive subject matter for this review focuses on the extreme thermoacidophiles from order Sulfolobales. The Sulfolobales comprise an order of archaea taxonomically defined within the class Thermoprotei, within the phylum Crenarchaeota. These organisms inhabit environments characterized by both extreme temperatures (65–90 °C) and low pH (1.0–3.5), such as terrestrial solfatara or mud pools, often closely associated with volcanic activity and laden with inorganic materials[12]. In fact, the first species of the order to be isolated, *Sulfolobus acidocaldarius* (from Locomotive Spring in Yellowstone National Park), was reported to oxidize sulfur to fuel autotrophic growth, leading to the name Sulfolobales[7]. However, this phenotype has not been observed in the currently studied *S. acidocaldarius* type strains, although many isolates from the genera *Sulfolobus, Metallosphaera* and *Acidianus* utilize S^0 as an electron donor[5,13–21]. Thus, reports that *S. acidocaldarius* strains from culture collections cannot[5] oxidize S° suggests that repeated passages on rich media have led to the loss of this ability or that inherent difficulties exist in isolating pure cultures from environmental enrichments. Beyond sulfur oxidation, several species, especially those from the genus *Acidianus*, *Sulfurisphaera*, and *Stygiolobus*, are capable of sulfur reduction, and often utilize hydrogen to produce hydrogen sulfide as a metabolic end-product[13,18,19,21–24].

While many members of the order grow lithotrophically, most known species exhibit modes of either strict heterotrophy or mixotrophy. Most members of the genera *Sulfolobus* and *Metallosphaera* are capable of utilizing protein-rich substrates, such as yeast extract or tryptone, under aerobic conditions. Furthermore, several species, such as *Sulfolobus solfataricus, Sulfolobus shibitae*[25], and *Sulfolobus islandicus*[26], use a wide variety of sugars in catabolic metabolism. In addition, members of the order, particularly in the genera *Metallosphaera* and *Acidianus*, are capable of oxidizing metal sulfides, a trait that is particularly useful for bioleaching of base, precious and strategic metals from mineral ores[27–30]. Finally, some members of the genus *Acidianus* are capable of using ferric iron as an electron acceptor under anaerobic conditions[21,31].

Carbon Dioxide Fixation—Interestingly, the natural habitats of many Sulfolobales (solfatara/calderas) are limited or devoid of complex carbon sources, necessitating the process of autotrophy. The ability of organisms to fix carbon dioxide from the atmosphere is considered by many to explain the early formation of the multi-carbon molecules required to fuel life, explaining their retention in species across all three domains of life[32,33]. As it stands, 6 major routes exist for the fixation of carbon dioxide: the Calvin-Benson cycle (present in most plants), the reductive citric acid cycle (green sulfur bacteria), the reductive acetyl-CoA cycle (acetogens/methanogens), the hydroxypropionate bi-cycle (*Chloroflexus*), and the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) or dicarboxylate/4-hydroxybutyrate pathways (both from the Crenarchaeota)[34]. The habitats from which the

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Sulfolobales were isolated, unlike many other organisms, is characterized by copious oxygen and inorganic electron donors[12]. While the reactions driving this cycle are some of the most energy-demanding for autotrophic carbon assimilation, their advantage may lie in their relative insensitivity to oxygen, avoidance of side-reactions, direct utilization of bicarbonate, and thermal stability[34,36].

Briefly, the 3-HP/4-HB cycle has two major products that enter into cellular metabolism. The first portion involves the addition of two bicarbonate molecules to acetyl-CoA to form succinyl-CoA, which is subsequently reduced in the second half of the cycle to 4-hydroxybutyrate and eventually dissociated to two molecules of acetyl-CoA (Figure 1)[37]. The key enzyme in the cycle is a biotinylated acetyl-CoA/propionyl-CoA carboxylase that is bi-functional and efficient in substrate turnover[38]. Metabolic analysis of the cycle has revealed that the major product of the cycle is not acetyl-CoA (as originally hypothesized), but rather succinyl-CoA (roughly two-thirds of the carbon flux), yielding malate and oxaloacetate in subsequent oxidation reactions[39]. This requires a turn and a half of the cycle to maintain acetyl-CoA levels and generate succinyl-CoA.

From an application-oriented point-of-view, it may be possible to use this pathway to sustainably produce high-value specialty chemicals, such as 3-HP or succinate. The former is used industrially in polymer production and the latter is used to produce solvents and polymers[40]. For this reason, several attempts have been made to utilize these genes in metabolically-engineered hosts. For instance, the first three enzymes have been expressed in *P. furiosus* to introduce a temperature-shift-responsive metabolic mode for the production of 3-HP[41]. Further work with this metabolically engineered strain has demonstrated that the addition of a biotin protein ligase can improve 3-hydroxy-propionate titers more than eight-fold[42]. This dramatic improvement is likely due to the presence of a biotinylated subunit on the key acetyl-CoA carboxylase enzyme from the cycle[37]. Thus, this well-studied pathway has opportunities to be utilized and improved upon.

Sulfur Utilization—In contrast to carbon metabolism, sulfur metabolic pathway discovery is hampered by the tendency of elemental sulfur and its derivatives to react non-enzymatically, masking the true identity of an enzyme's substrate or products[43,44]. Because S^0 is sparingly soluble in water under standard conditions, the true substrate for microbial growth on sulfur is likely soluble polysulfides and polythionates, introduced by non-enzymatic reactions[45–47]. However, these solubilizing reactions only occur at near-neutral pH, since under acidic conditions the equilibrium strongly favors elemental sulfur[44]. In order to overcome these solubility issues, it has been proposed that acidophiles may physically associate with sulfur particles or that at elevated temperatures sulfur becomes sufficiently soluble to support growth[47]. Regardless, these organisms have a suite of proteins capable of manipulating the initial elemental sulfur from the environment, as well as many of its derivatives.

The sulfur oxygenase reductase (SOR), first identified in a member of the Sulfolobales, *Acidianus ambivalens*[48], appears to be key to acidophilic sulfur oxidation. This intracellular enzyme is active on elemental sulfur, indicating transport of elemental sulfur or one of its derivatives to the cytoplasm by some still unidentified mechanism. SOR appears to be

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limited to the Sulfolobales and a few extremophilic bacteria[49]; this makes sense given that other sulfur lithotrophs grow closer to neutral pH where more soluble sulfur species are abundant. SOR acts on elemental sulfur by disproportionating it equally between oxidized (sulfite; SO_3^{2-}) and reduced (hydrogen sulfide; H_2S) products. Further, the production of thiosulfate observed in early studies of the SOR[48] is now believed to be the result of a non-enzymatic reaction[50]. SOR requires oxygen to function but uses no additional co-factors, suggesting its ability to conserve cellular energy. Instead, it "activates" long, unwieldy hydrophobic sulfur chains into smaller intermediates that can be used by other enzymes to generate energy.

Acidianus ambivalens has served as the model organism for sulfur metabolism studies in the Sulfolobales, since measurements of sulfur-active enzymes from cell extracts were used to construct a conceptual model of its sulfur oxidation pathways[51]. While the SOR enzyme has been the most thoroughly characterized enzyme with respect to sulfur metabolism[48,49,52], studies of enzymes purified or detected from *A. ambivalens* cell extracts provide some insights into the complete oxidation pathway. The *A. ambivalens* genome remains unsequenced, so many of its enzymes are identifiable only by their activity in cell-extracts. This presents a challenge for systems biology-based efforts to understand the details of sulfur oxidation in other Sulfolobales, or even how this process contributes to *A. ambivalens* energetics and metabolism. Regardless, there appears to be two parallel processes by which *A. ambivalens* (and presumably the other sulfur-oxidizing members of the Sulfolobales) gain energy while oxidizing elemental sulfur to sulfate (SO_4^{2-}). One pathway uses the membrane-associated oxidoreductases, TQO and SAOR, to reduce an electron carrier (such as quinone)[53], thereby generating proton motive force via the terminal oxidase[54,55], while the other pathway produces one high-energy phosphate bond (ADP from AMP) by direct substrate level phosphorylation via APSR and APAT, generating sulfate in the process[51] (Figure 2). While the sulfur metabolism has been examined in bioleaching applications (see next section), the ability of *S. metallicus* to remove toxic H_2S from high-temperature gas streams represents a potentially important technological use of sulfur oxidation[56].

A. ambivalens has also served as the model system for anaerobic sulfur reduction among the Sulfolobales[57]. The enzyme pathway for sulfur reduction in *A. ambivalens* appears to be much simpler than for oxidation, possibly involving only two closely associated membrane complexes. A membrane hydrogenase passes electrons (via a quinone molecule) from H_2 to a sulfur reductase, where they are used to reduce elemental sulfur to H_2S[57]. The cycling of quinones between the two enzymes – forming a "redox loop" similar to the one used in *Escherichia coli* during growth on nitrate[58] – is likely the way protons are transported across the membrane, coupling sulfur reduction to energy conservation (Figure 2).

Metal Oxidation—Along with interest in sulfur metabolism, some of the earliest work in determining the mechanism of metal oxidation in the Sulfolobales (and acidophiles, in general) involved the spectroscopic identification of unique cytochromes from iron-oxidizing cultures[59]. This original research led to the intensive study and eventual development of a model in the mesoacidophile *Acidithiobacillus ferroxidans*, involving the shuttling of electrons from the outer membrane of the cell to the inner membrane, driving a terminal oxidase to maintain pH homeostasis and the production of reducing power for

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intracellular metabolic needs[60]. Not surprisingly, the spectroscopic data from *S. metallicus* demonstrated early on that key differences exist between bacterial and archaeal metal oxidation, particularly in the presence of cytochromes and the roles of various protein complexes in transporting electrons[59,61]. However, some of the same systems-based approaches were utilized to detect the transcriptomic response of known iron-oxidizers, including *S. metallicus*[62], *M. yellowstonensis*[63] and *M. sedula*[64], in the presence of iron. Interestingly, these experiments suggest the importance of merging several systems biology techniques in order to ascertain new pathways and information. While all three species contain the *fox* stimulon (an assortment of ferrous-responsive genes A–J) and key related genes (such as rusticyanin and the cystathionin-β-synthase subunits A and B), their regulation varies dramatically among the species with both constitutive and inducible expression observed during iron supplemented growth[62–64]. Yet, the merger of this transcriptomic data and genomics analysis yielded a hypothesized pathway for metal biooxidation in these organisms (Figure 2), which relies on a cytochrome b (as opposed to a cytochrome c), bifurcating rusticyanin(s), and two terminal complexes: an NADH dehydrogenase (generating reducing power) and a putative cytochrome c oxidase (driving pH homeostasis). Although similarities exist between the two systems in *A. ferroxidans* and *Metallosphaera/Sulfolobus spp.*, distinctive co-factors and apparent differences in organization suggest that these systems are evolutionarily divergent modes of biooxidation[65].

These differences, as well as the major phenotypical differences between these two classes of metal mobilizers, relates to their use in metal bioleaching applications. For example, many mesophilic organisms are ill-suited to bioleaching of highly gangue (i.e., high sulfur content) ores due to the extremely exothermic nature of sulfur oxidation chemistry. The build-up of heat can be problematic in large heap operations that rely on mesophiles alone[66,67]. This physiological trait cannot be undervalued given that the removal of elemental sulfur can improve cyanidation (a form of chemically-driven mineralization), which is commonly used in gold mining. Furthermore, extreme thermophiles appear to have some niche advantages over mesophiles for bioleaching of several specific types of copper ores, including the enhanced dissolution of copper from recalcitrant primary ores (such as chalcopyrite)[68–70], selective mobilization of copper over molybdenum in copper-bearing molybdenite[71,72], and the unassisted mineralization of arsenic in the form of arsenate from enargite ores[73]. Bioleaching operations targeting copper have increased dramatically and currently account for more than 15% of the global output[74]. Thus organisms that present an inherent propensity for copper solubilisation such as *A. brierleyi, S. metallicus,* or *M. sedula* deserve more investigation for their potential industrial application.

Pyrococcus furiosus

Pyrococcus furiosus, the type strain of the genus, was first isolated in 1986 from a hydrothermal vent off of the coast of Vulcano Island (Italy) and has been one of the most studied hyperthermophiles to date, due to its intriguing phenotypical characteristics[75]. Exhibiting optimal growth at 100°C and a pH near 7, it was the second genus, after the autotrophic sulfur-oxidizing *Pyrodictium*, capable of growth at temperatures at or above 100°C[75]. As a heterotrophic organism, *P. furiosus* is capable of utilizing hexose

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oligosaccharides such as cellobiose and laminarin[76], chitin[77,78], and peptides[79]. Efforts over the past three decades have elucidated many unique features of this organism, including various novelties in metabolic pathways, regulatory mechanisms and proteins and enzymes.

Central Glycolytic Metabolism—*P. furiosus* grows well on disaccharides (maltose and cellobiose) and glucans (laminarin and starch), but not on monosaccharides nor pentoses[80]. The reasons for this anomaly are unknown but monosaccharides may not be available externally in these high temperature environments as they are susceptible to the Maillard reactions, in which sugars react with available amino acids to form glycosylamines; this problem is especially exacerbated in peptide-rich media. Disaccharide and polysaccharide transport may also be more efficient energetically.

P. furiosus derives no net substrate level phosphorylation from glucose to pyruvate conversion, unlike the traditional Embden-Meyerhof (EM) pathway that provides two ATP per glucose and the Entner-Doudoroff (ED) pathway which yields one ATP per glucose (see Figure 3). Thus, the only net substrate level phosphorylation gains are a result of ATP-forming hydrolysis of acetyl-CoA, produced from pyruvate via pyruvate oxidoreductase, by acetyl-CoA synthetase[81]. *P. furiosus* contains a non-traditional variation of Embden-Meyerhof glycolysis, in which glucokinase and phosphofructokinase utilize ADP as the phosphoryl group donor, generating AMP[80]. In the early 1990s, these were the first reported ADP-dependent kinases[80,82].

The absence of an energy-conserving step in the glycolytic pathway is due to the absence of a 1,3-biphosphoglycerate intermediate, which is found in both the EM and ED pathways. As shown in Figure 3, this direct conversion from glyceraldehyde-3-phosphate (GAP) to 3-phosphoglycerate (3PG) does result in production of a reducing equivalent in the form of reduced ferredoxin, but does not result in substrate level phosphorylation. The phosphate group is released via hydrolysis without capture of this high-energy bond. The enzyme responsible for the conversion of GAP to 3PG, GAP ferredoxin oxidoreductase (GAPOR), is unusual in that it requires tungsten, an element rarely found in biology[83]. The absence of other, more traditional glycolytic enzymes makes GAPOR's function critical to sugar utilization. Thus, tungsten levels have a significant impact on the growth of *P. furiosus* in the presence of maltose[83].

Fermentation Pathways—*P. furiosus* produces reduced ferredoxin through central glycolysis and, as an obligate anaerobe, must have a route to dispose of any excess reducing power. Two primary routes exist for this purpose depending on hydrogen partial pressures, the availability of elemental sulfur and nitrogen, and other regulatory factors. The primary route of regenerating oxidized ferredoxin is through a membrane-bound hydrogenase (MBH) that produces a ion gradient that allows ATP production via ATP synthase[84]. The hydrogenase is thought to exchange the proton gradient generated by hydrogen production for a Na^+ gradient and this is utilized by a Na^+-dependent ATP synthase[85]. This energy conserving hydrogenase therefore constitutes a single-step electron transport chain, and has been proposed as an evolutionary precursor to the complicated, multi-step electron transport chains that are common in present day microbes[81]. While the exact mechanism coupling proton transfer and hydrogen production is unknown, it is estimated that 0.3–0.4 molecules

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of ATP are produced per two electrons transferred[81]. Thus approximately 1.2 moles of ATP are produced for every mole of glucose converted to acetate via glycolytically produced reducing equivalents. Given the low energy production resulting from glycolysis, this fermentative process is particularly critical[81].

When elemental sulfur is present, *P. furiosus* produces hydrogen sulfide rather than hydrogen gas[86]. As with H_2 production, a proton gradient is formed by a membrane bound oxidoreductase (MBX)[87]. MBX is highly homologous to the membrane-bound hydrogenase and is thought to oxidize ferredoxin[87]. However, it is not known if MBX reduces sulfur directly or generates NADPH that is then used by a cytoplasmic NADPH- and CoA-dependent enzyme[87]. The reason for the preference for sulfur over proton reduction is not clear but it is strong since the switch from H_2 to H_2S production begins only minutes after the addition of sulfur to a growing culture[88]. The shift is mediated by SurR, a redox-responsive transcriptional regulator that has been well characterized[89,90].

Another method for disposing of reductant during fermentation involves the transformation of pyruvate to alanine with the addition of available nitrogen[91]. This results in a major energetic penalty, however, as the pyruvate is not used to produce acetyl-CoA, which is responsible for the majority of ATP production. Thus, alanine pathway is only utilized when sulfur is absent and the hydrogen partial pressures are high[91].

Applied Biocatalysis and Metabolic Engineering—Prior to detailed knowledge of the *P. furiosus* genome and development of genetic manipulation methodology, early work focused on characterizing its novel enzymes, with an eye towards industrial applications. While the DNA polymerase from *Thermus aquaticus* (Taq) is the most widely known and utilized thermostable polymerase in PCR reactions, the *P. furiosus* DNA polymerase is considerably more thermostable and of higher fidelity, yet two-to three-fold lower processivity. Due to its 3′ to 5′ exonuclease proofreading activity, the polymerase exhibits a ten-fold reduction in error rate compared to the Taq polymerase[92]. Additionally, the NADP(H)-dependent hydrogenase (SH1) from *P. furiosus* is extremely thermostable and has a temperature optimum of 95°C[84]. It has been utilized in a renewable H_2 production *in vitro* system in which sugars are completely oxidized to CO_2 and H_2. A combination of pure enzymes comprising the pentose phosphate pathway (PPP) were used to convert sugars to CO_2 and the NADPH that is then produced is oxidized and H_2 is produced by SHI [84]. Many other enzymes of interest from *P. furiosus* have been purified and characterized[93], and include carbohydrate hydrolyzing enzymes, (e.g., α-amylase[94], amylopullulanase[94], endoglucanase[95], and β-glucosidase[96], and chitinase[77]) and proteases[97,98].

P. furiosus now has a facile genetic system which has led to efforts directed at metabolic engineering[99]. Earlier work on a related extreme thermophile, *Thermococcus kodakarensis*, a member of the same order as *P. furiosus*, the Thermococcales, paved the way for the *P. furiosus* genetic tools[100]. For *P. furiosus*, its high growth temperature and tolerance to cold shock opens up its use for hosting metabolic pathways from much less extreme thermophiles[101–103]. In fact, a novel temperature-shift strategy has been demonstrated that minimizes *P. furiosus* metabolism at sub-optima temperatures to direct energy to heterologous production formation[104]. As mentioned above, *P. furiosus* produces soluble

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hydrogenases, which can regenerate reducing equivalents from hydrogen gas[41]. These hydrogenases could allow metabolically engineered *P. furiosus* to use electrons from H_2 to produce highly reduced chemical products[41]. The insertion of three genes from the *M. sedula* 3-HP/4-HB carbon fixation cycle into *P. furiosus* demonstrated production of 3-hydroxyproprionate (3-HP) utilizing sugars and sequestering carbon dioxide for a portion of the molecule[41]. Another heterologous pathway expressed in *P. furious* utilized genes from three thermophilic organisms with optimal temperatures ranging from 65–75°C for the production of n-butanol[105]. With this alcohol pathway, significant diversion to ethanol was shown due to promiscuity of the aldehyde dehydrogenase enzymes[105]. The use of less thermophilic enzymes in heterologous biosynthetic pathways has provided insights into *P. furiosus* native metabolism at lower temperatures. For example, at 70–80°C, acetoin is produced as a major metabolic product. Along these lines, it was shown that the removal of acetolactate synthase in *P. furiosus* generates small amounts of ethanol as a metabolic end product, as pyruvate was directed toward acetate and eventually ethanol rather than acetoin[103]. In addition to its native abilities to utilize sugars, *P. furiosus* was engineered with a 16 gene cluster to oxidize carbon monoxide to carbon dioxide, producing H_2 and energy in the process[101]. Overall, the ability to engineer utilization of unique energy sources, manipulate temperatures to optimize enzyme activities, and insert genes from a variety of organisms with different optimal growth temperature provide tools not typically available in model mesophilic hosts.

Caldicellulosiruptor spp

Caldicellulosiruptor is a bacterial genus containing the most thermophilic, cellulolytic microorganisms known to date. Isolated worldwide and having optimal growth temperatures between 70–78°C, these Gram-positive, asporogenic, obligate anaerobes have the ability to degrade unpretreated lignocellulosic biomass, a highly sought after phenotype for consolidated bioprocessing of fuels and chemicals. Many well-studied cellulolytic microbes are known to either secrete individual enzymes or large cellulolytic enzyme complexes (e.g., the cellulosome[106]) into their environment. In contrast, *Caldicellulosiruptor* species instead use an array of multi-modular enzymes to breakdown plant biomass[107–112]. These carbohydrate active enzymes (CAZymes) are composed of both catalytic (e.g., glycoside hydrolases [GH]) and non-catalytic (e.g., carbohydrate binding modules [CBM]) domains. All *Caldicellulosiruptor* species are able to utilize fructose, galactose, glucose, xylose, and pectin via a classical EMP pathway[113–115]. However, arabinose, rhamnose, and fucose utilization, is not conserved throughout all species[113,115–118]. Some of the sugars, such as xylose and arabinose, are broken down via the non-oxidative pentose phosphate pathway (PPP) and then piped into the EMP pathway as intermediates[119]. Although *Caldicellulosiruptor* species lack the oxidative PPP, which generally is responsible for NADPH production, they are still capable of generating NADPH; the exact enzymatic mechanism for this process is currently unknown[120]. Members of the *Caldicellulosiruptor* genus also contain an incomplete tricarboxylic acid (TCA) cycle, consisting of a reductive branch leading to fumarate and an oxidative branch producing succinyl-CoA.

Carbohydrate Utilization—*Caldicellulosiruptor spp.* produce many highly versatile and efficient multi-modular carbohydrate-degrading enzymes, made up of combinations of

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glycoside-hydrolase (GH) and carbohydrate binding module (CBM) domains. For example, CelA, a lignocellulosic CAZyme, is composed of five carbohydrate-specific domains: GH9-CBM3-CBM3-CBM3-GH48 connected by linker regions[121–123]. These different segments allow proteins to have multiple functions: simultaneously binding to its substrate (via the CBM3s), as well as cleavage of specific bonds (via the GH9 and GH48 domains); these GH9 and GH48 domains are capable of endo- and exoglucanase activity. CelA is present in only the most cellulolytic species of the genus and its two GH domains provide these species with a unique 'drilling' mode of action during biomass deconstruction[121]. While CBMs allow the CAZyme complex to adhere to the biomass, surface layer homology (SLH) domains are also found in the *Caldicellulosiruptor* multi-modular scheme[124,125]. Instead of being freely transported out of the cell, CAZymes with SLH domains are tethered the cell's S-layer. As such, the enzymes can breakdown and bind, if they contain CBMs, substrates in close proximity to the microbe, providing better access to available sugar moieties. It also has been recently found that *Caldicellulosiruptor* species have a novel method of attaching themselves to crystalline cellulose. Structurally unique proteins, called tāpirins, are expressed on the cell surface, and contain a binding domain specific to insoluble cellulose[126]. Present in every member of the genus and highly expressed, tāpirins are thought to play an important role in how plant matter is deconstructed by microbes this genus.

Both methods of attachment, along with the large inventory of glycolytic enzymes, give this genus its impressive ability to degrade a wide variety of lignocellulosic substrates[106]. *Caldicellulosiruptor* species are capable of breaking down cellulose and hemicellulose (hexoses and pentoses), both as simple monosaccharides and complex biomasses[108–110,116,127–131]. Unlike many cellulolytic organisms, they do not exhibit carbon catabolite repression, a process by which certain sugars are preferentially metabolized, while excluding the usage of others[106,111,114]. This is especially advantageous in an industrial process involving lignocellulose conversion to fuels and chemicals, as these microbes can utilize multiple sugars simultaneously, with numerous points of entry to central carbon metabolism (see figure 4). Although *Caldicellulosiruptor saccharolyticus* was shown to grow well on a variety of sugars (arabinose, fructose, galactose, glucose, mannose, and xylose) simultaneously, the extent of which each monosaccharide was digested varied, with fructose being the most utilized[111]. In the absence of an apparent carbon utilization regulatory system, variation in sugar utilization among *Caldicellulosiruptor* species is likely due to presence or absence of certain metabolic pathways, e.g. the oxidative PPP, and/or essential transporters; the latter has only recently been better understood for a few *Caldicellulosiruptor* species with transcriptomics analysis of growth on substrates, such as simple sugars, crystalline cellulose (Avicel), and complex biomasses like switchgrass[107,108,111,120,132].

One option to improve degradation of lignocellulose is to increase the CAZyme inventory of a microbe. While generally highly conserved in the *Caldicellulosiruptor* genus, the SLH domain xylanase from *Caldicellulosiruptor kronotskyensis*, Calkro_0402, is not present in *C. bescii* and, thus, was inserted into the genome to improve its ability to utilize xylan[125]. The manipulated strain successfully expressed the protein on the S-layer of the cell and improved xylan utilization significantly by doubling xylose release into the supernatant from oat spelt

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xylan. Growth on washed and unwashed birch xylan was improved, while dilute acid-pretreated switchgrass solubilization remained unaffected; this indicated that there are still other hurdles to lignocellulosic degradation that must be overcome. However, on substrates with high xylan content, the engineered strain showed improved solubilisation, possibly due to increased substrate attachment.

Fermentation—The major fermentation products of the *Caldicellulosiruptor* genus are hydrogen, carbon dioxide, and acetate[116,133,134]. Lactate production has also been measured, but only a trace amount of ethanol has been detected in wild-type cultures[113,117,118,135]. By far, the most studied product of all of these is molecular hydrogen, especially with *C. saccharolyticus*[114,120,136–149]. H_2 generation is completed via hydrogenases utilizing reducing equivalents (Fd^{red} and NADH) from central carbon metabolism (see figure 4). *C. saccharolyticus,* along with several other extreme thermophiles, is considered to be an ideal 'biohydrogen factory', as reported yields are close to the so-called 'Thauer limit' of 4 moles H_2 per mol glucose[114,116,150]. Decreased H_2 production results in the accumulation of NADH and Fd^{red}, while increased H_2 can instead push metabolic flux toward lactate production. At high levels of molecular hydrogen, product inhibition occurs via increased dissolved H_2 levels[151]. NADH and Fd^{red} are simultaneously oxidized by a bifurcating [FeFe]-hydrogenase, which uses both electron donors at the same time[152], while Fd^{red} is also oxidized by a membrane-bound [NiFe]-hydrogenase that is related to that found in *P. furiosus*[85].

With the recent development of a genetic engineering system in *Caldicellulosiruptor bescii*, based on auxotrophic selection, targeted manipulations of the *Caldicellulosiruptor* genome and consequently metabolism are now possible[153–156]. The first directed demonstration of these methods actually involved the deletion of the single lactase dehydrogenase (*ldh* - Athe_1918) present in *C. bescii* to halt lactate production[157]. While the wild type and parent strains produced less hydrogen than the well-studied *C. saccharolyticus* (1.8 and 1.7 vs 2.5 mol H_2/mol of glucose, respectively), the *ldh* knockout produced significantly more H_2 on switchgrass, closer to the theoretical goal (3.4 mol H_2/mol of glucose). As lactate formation ceased, acetate production increased by 38–40% over the wild type and parent strains.

More recently, ethanol production was demonstrated in *C. bescii* through the addition of an NADH-dependent alcohol dehydrogenase gene from *Clostridium thermocellum* (*adhE* – Cthe_0423) into the strain lacking lactate formation[158]; *C. bescii* does not possess a native alcohol or acetaldehyde dehydrogenase, and thus a representative gene was recruited from another thermophilic Firmicute. Due to the lower thermostability of the *C. thermocellum* protein, growth of the engineered strain was done at a maximum of 65°C. Strain growth on cellobiose, Avicel and switchgrass, and resulted in 14.8 mM, 14 mM, and 12.8 mM ethanol, respectively. Acetate production was also lowered, ranging from ~4–5 mM compared to the wild type (~6 mM) and parent (~8–9 mM) on all tested substrates. Another attempt at ethanol production was completed by individually inserting two bi-functional alcohol dehydrogenase genes from *Thermoanaerobacter pseudethanolicus* 39E, *adhB* (Teth39_0218) and *adhE* (Teth39_0206), into the *C. bescii* lactase dehydrogenase knockout[159]. Growing the modified strain at 75°C with cellobiose, ethanol was produced at reported levels of 1.4 mM and 2.9 mM, acetate at 15.5 mM and 14.1 mM, and H_2 at 23.2 mM and 22.5 mM for the

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AdhB and AdhE knock-in strains, respectively; similar ethanol levels were also measured on Avicel and switchgrass.

With a genetic system now in place, gene 'knockouts' in *C. bescii* can be strategies to understand *Caldicellulosiruptor* metabolism and physiology. For instance, an uncharacterized [Ni-Fe] hydrogenase maturation gene cluster (*hypABFCDE* – Athe_1088–Athe_1093) was deleted from the aforementioned, modified ethanol-producing *C. bescii* strain containing *adhE* from *C. thermocellum*[160]. The resulting strain produced 20% less H_2 than its parent, yet its H_2 yield per mol of cellobiose increased 63% (3.58 vs 2.19 mol H_2/mol cellobiose). Fermentation patterns on Avicel, cellobiose, and switchgrass showed that the engineered strain also produced acetate (1.6–5.7 mM – 34% less than parent) and ethanol (1.9–2.7 mM – 73% less than parent). Additionally, the knockout had reduced growth and a longer lag phase, which could result from the deleted gene acting as an ATP-generating protein pump. Other genetic manipulations with *C. bescii* include the deletion of CelA (Athe_1867)[123], a predicted pectin lysase, and a putative AraC family transcriptional regulator genes (*pecABCR* – Athe_1853–1856)[161]. Continued efforts with the newly established genetics tools in *C. bescii*, and eventually other *Caldicellulosiruptor* species, will help reveal the basis for its ability to grow on lignocellulosic substrates.

Thermotoga spp

The bacterial genus *Thermotoga* contains nine named species that are obligate anaerobes capable of growth at optimal temperatures between 65–80°C, mostly isolated from submarine geothermal features[162–164]. These rod-shaped, Gram-negative, eubacteria were originally identifiable by their distinctive 'toga'-like outer sheath and absence of an outer membrane. Beyond their unique appearance, the species in the genus *Thermotoga* share a remarkably large number of homologs (roughly 24% of the genome) with sequenced archaea[165]. This curious result has led some research into the evolutionary divergence/convergence of this bacterial lineage, suggesting the genomic features that may be critical in defining thermophily, such as the discovery of genes associated with biosynthesis of di-myo-inositol-phosphate, which may serve as a critical thermoprotectant compatible solute[166]. Further phylogenetic analysis has even suggested that mesophily may have developed from thermophily (within the order Thermotogales), given the ancestral sequence reconstruction of more thermally stable myo-inositol-phosphate synthase (MIPS)[167] and emergence of 'mesotoga' species[168]. Also of interest is the presence of a system for catabolizing *myo*-inositol that provides utilization of compatible solutes but cannot provide a complete source for carbon utilization[169]. Within the genus, *Thermotoga maritima* has served as a model species for studying evolution, biomass deconstruction, and biohydrogen production[170].

Carbohydrate Utilization—All *Thermotoga* species are chemoheterotrophs, although the range of substrate usage varies and includes numerous pentoses, hexoses, disaccharides, and polysaccharides, as well as yeast extract, acetate, methanol, and pectin[163,164,171–175]. This ability to utilize a broad array of carbohydrates appears to be supported by bioinformatics and transcriptomics suggesting a substrate-specific regulation and function of large numbers of ABC-transporters[176], as well as many α- and β-glycoside hydrolases (GH)[112]. Intriguingly, *T. maritima* grows faster on complex carbohydrates than on monosaccharides,

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suggesting an adaptation to the breakdown of biomass in their natural environments[177]. The metabolism of carbohydrates by these organisms results in the formation of some typical fermentation products, such as acetate, carbon dioxide, and lactate, but also the generation of molecular hydrogen, and small amounts of ethanol, butanol, and butyrate[178]. It is worth noting that *Thermotoga* species utilize the traditional EMP and ED pathways[179,180] for carbon utilization. However, they also contain, in some cases, unique enzymes that are adapted to optimizing the use of reducing power and energy generated from biomass deconstruction for the synthesis of fermentation products.

The initial genome annotation of *T. maritima* suggested a prevalence of mono- and polysaccharide utilization proteins (as much as 7% of identified genes)[165]. In contrast to organisms that produce large complexes for carbohydrate degradation (i.e., cellulosomes), *T. maritima* utilizes a broad array of both extra- and intra-cellular glycoside hydrolases, which have been detailed in previous reviews[112]. More recent examination of the pan genome, as well as transcriptomic data, suggests that *Thermotoga* species vary with respect to specific ABC sugar transporters and glycoside hydrolases[181]. Overall, the preponderance of thermally-stable, polysaccharide-degrading enzymes makes members of the genus and their enzymes intriguing candidates for the deconstruction of complex carbohydrates in industrial applications[182]. However, one of the limiting factors is the absence of any apparent capacity for growth on crystalline cellulose, suggesting a lack of cellulolytic enzymes in *Thermotoga* species[183]. In fact, to address this issue, efforts were directed at the ectopic expression of cellulases from *C. saccharolyticus* fused with *T. maritima* signal peptides. The resulting plasmids were used for *Thermotoga sp.* strain RQ2 transformations, where enhanced exoglucanase activity was observed, but eventually was lost due to poor plasmid maintenance[184]. However, a stable genetic system for *T. maritima* and *T. sp.* RQ7 was recently reported, based around a cryptic plasmid isolated from the latter[185].

Fermentation—Of the major fermentation products from *Thermotoga spp.*, H_2 production is particularly interesting from a biotechnological perspective. High yields (3.8 mol H_2/mol glucose) reported by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions[186–188] approach the Thauer limit[150]. The production of H_2 is most efficient when the balance of fermentation products is skewed toward acetate production as compared to lactate production, given that the enzymes identified in acetate production, phosphate acetyl-transferase and acetate kinase, avoid the re-oxidation of NADH and instead produce Fd^{red} and ATP, respectively. In contrast, the production of lactate is driven by a lactate dehydrogenase that uses reducing equivalents (NADH) generated in the glycolytic process. Another possible key to efficient hydrogen production in these organisms, as in the *Caldicellulosiruptor*, is the coupling of Fd^{red} and NADH oxidation by a bifurcating, [FeFe] hydrogenase[189], in which Fd^{red} likely drives the less favorable oxidation of NADH and improves the overall thermodynamics for producing hydrogen. This enzyme complex, first identified in *T. maritima*, appears to have a homolog in *T. neapolitana,* which has the highest reported H_2 yields within the genus. There is also evidence that the build-up of molecular hydrogen and a possible inhibition mechanism can be alleviated through the co-culturing of *T. maritima* with *Methanococcus jannaschii;* the latter oxidizes H_2 and generates

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methane[190]. This results in significant up-regulation of carbohydrate-active enzymes and growth-phase enzymes, as well as denser cultures[191] (see Figure 5).

Besides molecular hydrogen, several species have been reported to produce ethanol as a fermentation product[172,192]. This result was not expected given the lack of detectable pyruvate decarboxylase activity. However, more recent work has identified the presence of both an alcohol dehydrogenase (from *Thermotoga hypogea*)[193] and a bi-functional pyruvate ferredoxin oxidoreductase-pyruvate decarboxylase enzyme[194]. Additionally, butyrate (an odiferous organic used primarily as a perfume or food additive) production has been linked with hydrogen biosynthesis in studies involving *T. neapolitana*[195]; however, the mechanism of butyrate synthesis is still unknown in these organisms.

Thermus spp

The genus *Thermus* was among the first bacteria to be studied with respect to thermophily (Oshima and Imahori 1974), and the DNA polymerase from *Thermus aquaticus*[2] was used in early efforts with the polymerase chain reaction (PCR) (ref). While not all *Thermus* species (at least 11 have been named and characterized) grow optimally at 70°C and above (extremely thermophilic), there are some that meet this thermal threshold. *Thermus* species are typically non-motile, non-sporulating, and are not naturally capable of fermentation. Efforts directed at understanding *T. thermophilus* metabolism revealed that this bacterium uses glycolysis and the TCA cycle to drive carbon flux and bioenergetics (Lee et al., Microb Cell fact 2014; Swarup et al. Metab Eng 2014). Molecular genetics tools were developed for *Thermus thermophilus* (Koyama Hoshino 1986 J Bact), based on its natural competence, which opened up opportunities for it to be examined as a model thermophile. In fact, the relative stability of thermophilic enzymes and early interest in the genus sparked the undertaking of crystallization projects aimed at characterizing recombinant versions of all the identified coding ORFs from *Thermus thermophilus* (Yokoyama et al. 2000; Sazanov and Hinchliffe 2006; Selmer et al. 2006; Severinov 2000; Yusupov et al. 2001). The overall goal of such projects was to provide a comprehensive database of structural characteristics that aid in the determination of protein function and domain architecture representing all of the major classes of proteins identified to date.

Enzyme and metabolic engineering efforts with *Thermus*—Although more thermophilic microorganisms have become available, *Thermus* species can be sources of thermostable enzymes for biotechnological applications. For example, enzymes from *Thermus* were included in an *in vitro* pathway that converted glucose into n-butanol (Krutsakorn Metab Engr 2013) and a xylose isomerse from this species was used to enable a recombinant *Saccharomyces cerevisiae* strain to grow on xylose (Karhumaa et al. Yeast 2005). Although genetics are relatively facile for these organisms, metabolic engineering pursuits have been limited. One of the earliest examples of metabolic engineering of the organism involved the transfer of nitrification genes among two members of the genus, allowing an aerobic *Thermus* species to grow anaerobically (Ramirez-Arcos, 1998). Additionally, a few attempts have been made at overexpression of native genes for the purpose of biotechnological applications involving specific enzymes such as DNA polymerase and Mn-dependent catalases (Hidalgo 2004; Moreno 2005). More recently, a

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strain of *T. thermophilus* HB8 was generated that could co-utilize xylose and glucose at temperatures up to 81°C, with a view towards processing lignocellulose, although this strain could not deconstruct biomass nor ferment the C5/C6 sugars (Cordova et al., 2016).

Conclusion

Although the study of extreme thermophiles has only gained traction in the past few decades, there are numerous metabolic and physiological features that distinguish these organisms from the other major groups of life and justify continued research endeavours. Much of this information has been ascertained via the use of genomics, transcriptomics, and proteomics in conjunction with traditional microbiological/biochemical techniques. Furthermore, this synthesis has led to the development of metabolic and physiological models in extreme thermophiles that are beginning to rival better characterized mesophilic systems. With the advent of next-generation sequencing technologies, it seems likely that previous work will be furthered by large-scale comparative genomics and metagenomics projects; this should further the discovery of novel metabolic features (i.e. enzymes and native biological pathways) with vital importance to our fundamental understanding of biology.

Beyond the scientific merit of studying extreme thermophiles, numerous opportunities exist to utilize these organisms for biotechnological advancement. As previously emphasized, the extreme conditions under which these organisms subsist has led to evolutionarily distinct metabolic and physiological features. In general, thermally stable proteins and heat-tolerant metabolic hosts could provide a major economic benefit to industrial processes. In the case of upstream processes, it may be possible to eliminate or minimize the energy costs associated with cooling or sterilizing bioreactors; while downstream processes may benefit from simple techniques -- such as heat pre-treatment -- to select for thermophilic enzymes produced recombinantly in mesophilic hosts, eliminating costly purification steps. Additionally, the increase in available genetic systems in these organisms will open many avenues for metabolic engineering. In fact, these organisms could have vital roles in the future of bioprocessing ranging from sustainable biochemical engineering to specialty chemical production to the deconstruction of inorganic and organic raw materials and even the recovery of base, precious and strategic metals.

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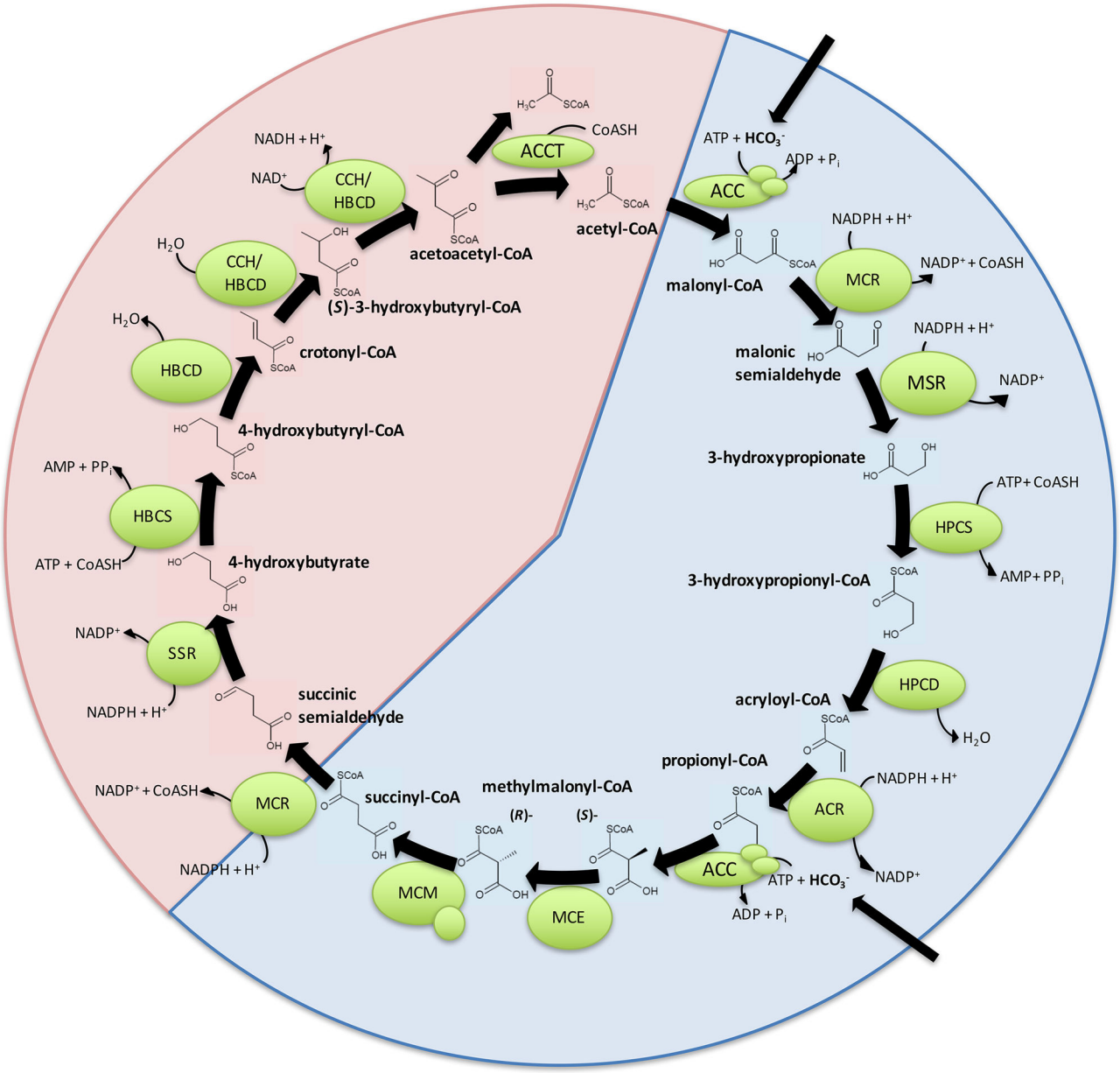

Figure 1. 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle from *Metallosphaera sedula*

The cycle consists of two major portions: carbon incorporation (via bicarbonate) occurs in the first half (blue) of the cycle and is followed by subsequent reduction and reformation of two acetyl-CoA molecules in the second half (red).. Enzymes listed and their references: acetyl-coA carboxylase[38,196] (ACC), acetoacetyl-CoA β-ketothiolase[37] (ACCT), acryloyl-CoA reductase[197] (ACR), crotonyl-CoA hydratase[198] (CCH), 4-hydroxybutyrate-CoA dehydratase[37] (HBCD), 4-hydroxybutyrate-CoA synthase[199] (HBCS), 3-hydroxypropionate-CoA dehydratase[197] (HPCD), 3-hydroxypropionate-CoA synthase[200] (HPCS), methylmalonyl-CoA epimerase[201] (MCE), methylmalonyl-CoA mutase[201] (MCM),

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malonyl-CoA/succinyl-CoA reductase[202] (MCR), malonate semialdehyde reductase[202] (MSR), succinate semialdehyde reductase[202] (SSR).

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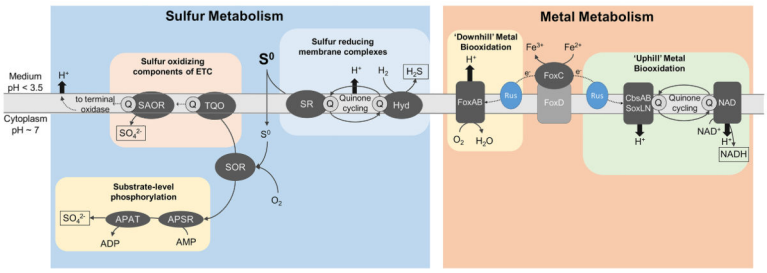

Figure 2. Chemolithotrophic pathways in the Sulfolobales

The first half of the figure (blue) shows the hypothetical pathways for sulfur utilization in the Sulfolobales, including both oxidizing and reducing pathways, beginning with elemental sulfur. Sulfur reducing complexes: hydrogenase (Hyd), sulfur reductase (SR)[57]. Sulfur oxidizing enzymes: sulfur oxygenase reductase (SOR)[48], thiosulfate:quinone oxidoreductase (TQO)[53], Sulfite:acceptor oxidoreductase (SAOR), adenylylsulfate reductase (APSR), adenylylsulfate:phosphate adenyltransferase (APAT)[51]. The second panel shows a hypothetical pathway for the oxidation of ferrous iron using several fox stimulon proteins as well as some iron-responsive respiratory proteins. Ferrous-Oxidation (Fox), rusticyanin (Rus), cystathionine-β-synthase containing protein subunits A and B (CbsAB), sulfur oxidation (Sox), NADH dehydrogenase (NAD)[62,64].

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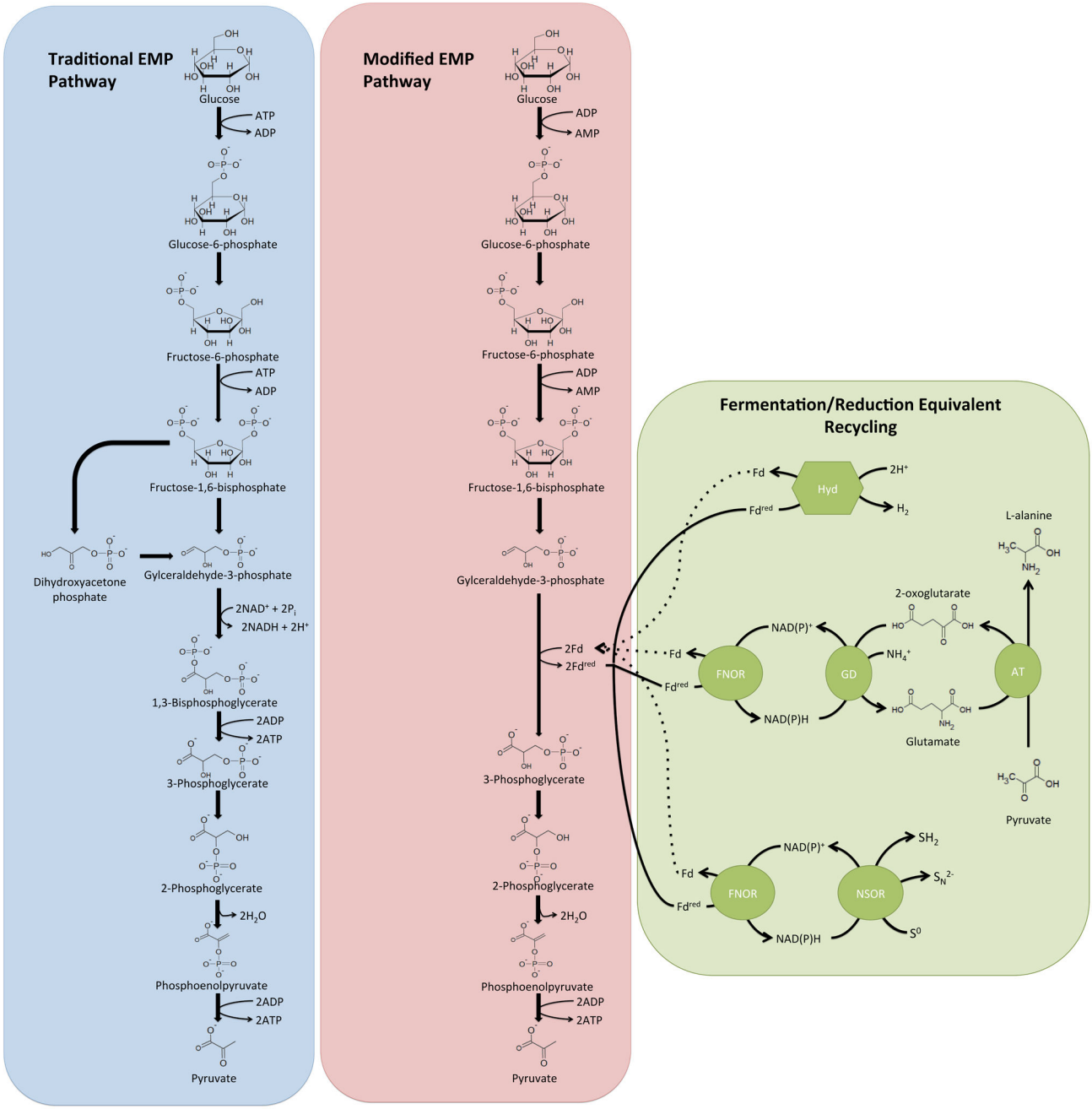

Figure 3.

Comparison of traditional Embden-Meyerhof-Parnas pathway with the modified pathway in the archaeon *P. furiosus*. **Included are three fermentative pathways which utilize the reduced ferredoxin produced via glycolysis. Enzyme abbreviations: hydrogenase (hyd), ferredoxin:NADP oxidoreductase (FNOR), glutamate deaminase (GD), alanine aminotransferase (AT), and NADP:sulfur oxidoreductase (NSOR).**

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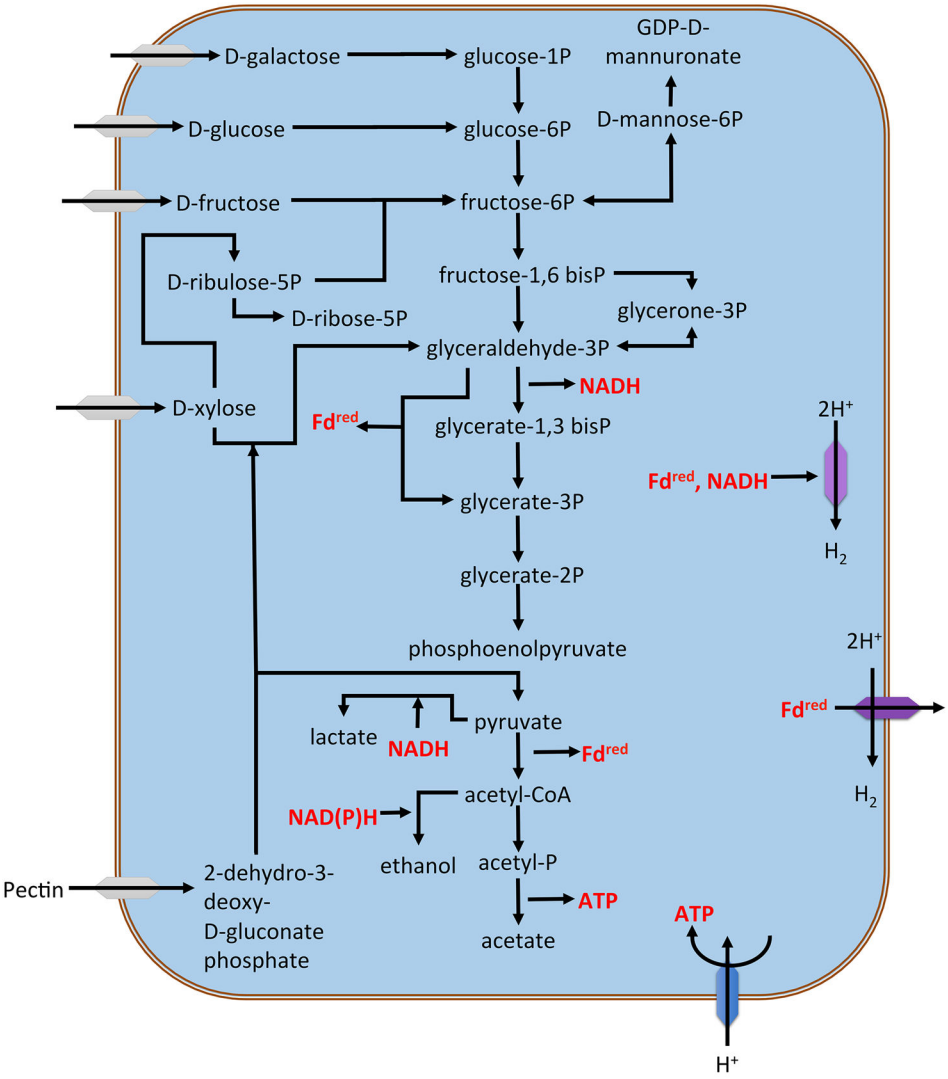

Figure 4. Conserved metabolic pathways in all *Caldicellulosiruptor* species

This includes sugar uptake, glycolytic, and fermentative pathways. The figure includes only the major steps, or start & end products. P is the abbreviation for phosphate, NADH for reduced nicotinamide adenine dinucleotide, ATP for adenosine triphosphate, Fd^{red} for reduced ferrodoxin, and GDP for guanosine diphosphate.

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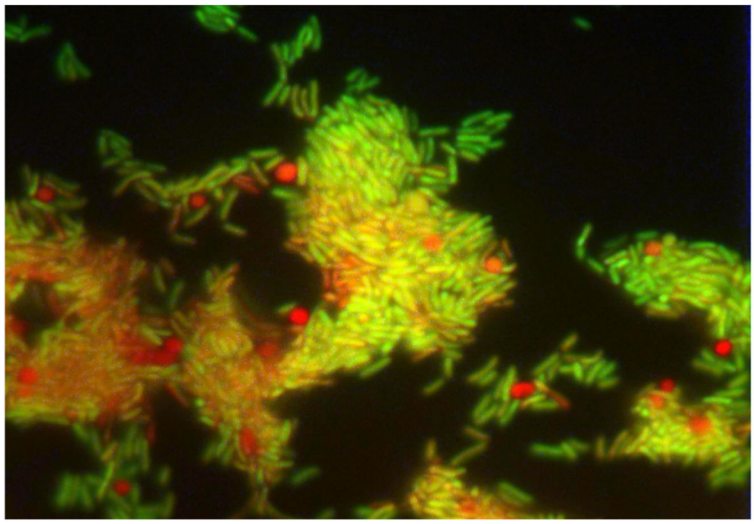

Figure 5.

Co-culture of *Thermotoga maritima* yellow/green rods) and *Methanocaldococcus jannaschii* (red cocci)[191] – permission pending.