Fall 8-28-2017

Examination of Contribution of Pentose Catabolism to Molecular Hydrogen Formation by Targeted Disruption of Arabinose Isomerase (*araA*) in the Hyperthermophilic Bacterium, *Thermotoga maritima*

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by

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A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In the Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor Paul Blum

Lincoln, Nebraska

August, 2017

Examination of Contribution of Pentose Catabolism to Molecular Hydrogen Formation by Targeted Disruption of Arabinose Isomerase (*araA*) in the Hyperthermophilic Bacterium, *Thermotoga maritima*.

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University of Nebraska, 2017

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Thermotoga maritima ferments a broad range of sugars to form acetate, carbon dioxide, traces of lactate and near theoretic yields of molecular hydrogen (H_2). In this organism, the catabolism of pentose sugars such as arabinose depends on the interaction between the pentose phosphate, Embden Myerhoff and Entner Doudoroff pathways. While values for H_2 yield have been determined using pentose supplemented complex media (CM) and predicted by metabolic pathway reconstruction, quantitative in vivo measurements derived from pathway elimination have not been reported reflecting the lack of a genetic method for the creation of targeted mutations. Here, a spontaneous and genetically stable *pyrE* deletion mutant was isolated and used as a recipient to refine transformation methods for its repair by homologous recombination. To verify the occurrence of recombination and to assess the frequency of crossover events flanking the deleted region, a synthetic *pyrE* allele was employed encoding synonymous nucleotide substitutions. Targeted inactivation of *araA* (arabinose isomerase) in *pyrE* mutant was accomplished using a divergent, codon optimized *T. africanus pyrE* allele fused to the *T. maritima groES* promoter. Mutants lacking *araA* were unable to catabolize arabinose in defined medium. The *araA* mutation was then repaired using targeted recombination. H_2 synthesis using CM supplemented with

arabinose was compared between wild type and *araA* mutant strains to provide a direct measurement of H_2 production dependent on arabinose consumption. Development of a targeted recombination system for manipulation of *T. maritima* provides a new strategy to explore H_2 formation and life at temperature extremes in the bacterial domain.

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ACKNOWLEDGMENTS

I would first like to give my great appreciation for the encouragement I have received from my family especially my late mother Gladys White. She was one of the main reason for me never giving up on my educational goal to earn my PhD. I would like to express my sincere thanks to my adviser Dr. Paul Blum for his support and throughout my PhD experience at UNL. I would like to extend my appreciation of my committee members, Dr. Black, Dr. Hutkins, Dr. Weber, and Dr. Buan for their support, advice, and encouragement during my PhD program. Furthermore I would like to give thanks to all faculty and staff from the School of Biological Sciences. I would like to thank all recent lab members, former lab members, recent postdocs, and former postdocs who made this PhD experience a fun and memorable one. I would like to give a special thanks to my colleague Raghuveer Singh who gave a lot of help on my project during my PhD program at UNL.

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CHAPTER 1

Literature Review

The need for new model microorganisms for biological H_2 production

High energy cost has increased due to several factors, mainly the depletion of oil, has opened up opportunities to investigate other options for the production of bio-fuels (1). Even though two chemical processes are used in the production of H_2 that depend on the burning of fossil fuels, the alternative route of formation of H_2 biologically in fermentative microbes is of great interest (2). The need for clean and renewable energy has called for other methods (namely microbes) that can produce hydrogen (which has become the target resource for clean energy) by utilizing carbohydrates or sugars. This ability demonstrated by hyperthermophiles to use biomass, which leads to hydrogen production, makes them conceivable models for biological manipulation.(3). A promising attribute of hyperthermophilic anaerobic organisms is their ability to thrive and enzymes remain thermo-stable at high temperatures (4).

In labs, microorganisms have become established as "research models" for many reasons, including ease of cultivation, familiarity with biochemical, genetic, metabolic, physiological, and ecological properties, genome sequence availability, and access to a genetic system. Promising microorganisms for specific scientific and technological purposes are often ignored as model systems because of existing limitations in one or more of these areas. The path of least resistance is to focus on existing models (e.g., *E. coli*),

instead of more appropriate choices that require the development of new tools and needed data. For biohydrogen production, members of the Thermotogales offer great promise as model fermentative anaerobes and, in recent years, have been studied extensively (5). In particular, *Thermotoga maritima* (6), the most studied member of the Thermotogales, has been genome sequenced (7), the target of a structural genomics effort (8), and examined comprehensively from a functional genomics perspective (9), (5), (10), (11), (12), (13), (14), (15), (16), (17). Furthermore, substantial genome sequence information is becoming available for 14 species within the Thermotogales (see partial list at http://www.genomesonline.org/), such that comparative genomics, using *T. maritima* as a basis, will soon be possible. To address a current limitation in studying the Thermotogales, efforts to develop a versatile molecular genetic system for *T. maritima*, which could be extended to other *Thermotoga* species, are proposed here. By establishing *T. maritima* and perhaps other Thermotogales as model microorganisms, unprecedented insights into the basis for biohydrogen production can be obtained and the advantages and disadvantages of thermophiles in this regard can be properly assessed.

Thermotogales

Base on the 16S rRNA (Fig. 1.1) tree generated by using the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence, Thermotogales constitutes the deeply rooted members on the phylogenetic tree (18). This phylum contains a single family, Thermotogaceae (Fig. 1.2), in which the genera *Thermotoga, Thermosipho, Fervidobacterium, Geotoga, Petrotoga, and Marinitoga* have been described (18). *Thermotoga* species have the capability of growth in temperature ranging from 55-90^{O}C and along with member of the order Aquificales represent a class of

bacteria that can endure such temperatures (6). Member of Thermotogales can be found in various extreme environments namely heated geothermal and volcanic vents (6). Even though they maintain an heterotrophic lifestyle, in their natural environment that play a role in consumption of microbial biomaterial (19). Members of Thermotogales can be characterized by the following features: i) there unique non-spore forming rod shape structure enclose in a proteinaceous sheath-like envelope called the "toga"; ii) Gram-negative strictly anaerobic fermentative bacteria (6). These bacteria obtain energy by fermentation of complex medium and simple sugars and excrete excess reductants in the form of hydrogen, acetate, carbon dioxide, and lactate (6, 20, 21).

***Thermotoga maritima*:**

The hyperthermophilic bacterium, *Thermotoga maritima* (*Tma*) is one of the many bacteria from Thermotogaceae and a strict anaerobic hyperthermophile (6). This unique bacterium was isolated from a hyperthermophilic geothermal vent in Vulcano, Italy(6). *Tma* optimum growth temperature is 80^{O}C, with an optimum pH of 6.5, and consumes simple and complex sugars to produce molecular H_2 as one of its by-products (5, 6, 20, 22). Out of the *Thermotoga* species, *Tma* and *T. neapolitana* have been extensively studied due to their ability to utilize a large array of polysaccharides that range from starch to pentoses and hexoses to produce molecular hydrogen (1, 5, 10, 20, 21). It has also been reported that *Tma* can consume pentose and hexoses simultaneously making this unique metabolism a very interest trait as it pertains to hydrogen production (5, 10). The presence of a catabolite repression mechanism have been reported for *T. neapolitana*, however *Tma* lacks this mechanism and it is still unknown whether other members of Thermotogales harbors this mechanism (23). Carbohydrate utilization by *Tma* differs from archaeal

species such as *Pyrococcus furiosus* in that it uses classic Embden-Meyerhof-Parnas (EMP) pathway to ferment hexose but lacks the ability to utilize peptides as the sole carbon and energy source (24, 25). *Tma* can convert glucose completely to organic acids (mainly acetate and lactate), CO_2, and H_2 (6). These process is carried out mainly through the EMP pathway (85%) with the remaining carbon flux converted via the Entner-Doudofoff (ED) pathway (15%). (Fig 1.3) Since they consume glucose efficiently, *Tma* displays a high specific H_2 production rate, a characteristic that is exhibited by hyperthermophilic archaeal *Thermococcus* species which allows *Tma* to reach the Thauer limit of 4 mol H_2 produced per mol hexose (Table 1.4) consumed (22). The generation of excess reductant via this pathway Fig. 1.3 (2 moles of NADH and reduced ferredoxin), it is imperative that *Tma* transfers these electrons using the FeFe hydrogenase (20). The production of H_2 however leads to the inhibition of many cellular processes which in turns leads to reduction in biomass formation (11, 26, 27). The H_2 inhibitory affects also leads to a shift in metabolism in which lactic acid production is activated by the lactate dehydrogenase in an efforts to remove excess reductant (20, 26). There has been significant effort to understand H_2 accumulation when *Tma* is cultivated on hexoses (oxidative pathway), but recently there has been reports on H_2 production when pentose are utilized (21). With a sequenced genome, transcriptional studies, a genetic system, along with functional genomic analysis makes *Tma* a model organism to evaluate the role of non-oxidative pentoses on H_2 production

Thermodynamics of fermentative hydrogen production:

The fermentation of glucose (and related carbohydrates) to H_2 has been closely examined in model mesophilic bacteria, mostly facultative anaerobic enterics, such as

Enterobacter aerogenes (28), *Enterobacter cloacae* (29) and *Escherichia coli* (30), and various Clostridia (31). In principle, four molecules of H_2 can be produced from the complete breakdown of one molecule of glucose through a traditional Emden-Meyerhof-Parnas (EMP) pathway (32):

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+$$

The free energy yield of this reaction ($\Delta G'_0$ = -206 kJ/mole) is sufficient to support microbial growth. Theoretically, this maximum yield of 4 (called the "Thauer limit" (22) assumes that common electron carriers, such as NADH, reduced ferredoxin, and formate, can transfer electrons to protons and form molecular hydrogen. The actual yields of this pathway are usually lower for two reasons. First, the hydrogenase-catalyzed formation of H_2 is affected by the type of intracellular electron carriers and can be bottlenecked by accumulation of H_2. For example, the reduction potential of ferredoxin is typically near that of H_2 (-420 mV, pH 7.0), which is the same as that for formate; these potentials are much better electron donors than NADH (E_{NADH} = -320 mV). Furthermore, in a metabolic setting, the impact of H_2 partial pressure on H_2 formation is related to the redox potential of electron carriers. The thermodynamically favorable partial pressure limit of H_2 ($P_{H2, max}$) has been estimated as follows (3):

$$P_{H2, max} < \exp (2F(E_{H2}-E_x)/RT)$$

where F is the Faraday constant, R the universal gas constant, and T the absolute temperature. Thus, if the redox potential of the electron carrier differs significantly from H_2, relatively low H_2 partial pressures will become self-limiting. If formate is used as the electron carrier, H_2 accumulation will have minimal thermodynamic impact. However, H_2

production ceases at partial pressures of 0.3 atm and 6 X 10^{-4} atm when ferredoxin (assuming an E_m value of – 400 mV) and NADH are used as electron donors, respectively. The second reason that yields fall below the Thauer limit is that there are other intracellular reactions involving NADH as an electron donor, i.e., H_2 is not the only sink for electrons. Furthermore, the formation of reduced ferredoxin and formate depends on the decarboxylation of pyruvate and this important metabolic intermediate can be diverted into either anabolism or metabolic byproducts, such as lactate and alanine (33). The key issue with respect to the Thauer limit is whether thermophiles use different metabolic strategies than mesophiles for electron flux related to carbohydrate fermentation and, if so, do these offer potential advantages for biohydrogen production.

Pentose Phosphate Pathway (PPP)

The pentose phosphate (PP) pathway is a metabolic pathway (Fig. 1.3) equivalent to glycolysis which functions to generate NAPDH and pentoses including ribose 5-phosphate the precursor for synthesis of nucleotides (34, 35). It also plays a role in the synthesis of required essential metabolites such as amino acid and vitamins as wells as constitutes of the lipopolysaccharide layer (34, 36). Unlike glycolysis, oxidation of glucose in the PPP role is anabolic instead of catabolic (34). The PPP consist of two different pathways the oxidative branch and non-oxidative branch (34). In the oxidative branch of PPP, the first step is formed by the enzyme glucose-6-phosphate followed by a three step process that leads into glycolysis (34). The non-oxidative branch plays a role in the generation of fructose-6-phosphate and glyceraldehyde-3-phosphates via the interconversion of 3 to 7 sugars which in turns enters the EMP pathway (34). In *E. coli* the utilization of pentoses such as xylose, ribose, and arabinose can only enter via the PP

pathway (36). For the non-oxidative PP pathway, there are 5 steps that occurs for the generation of the 3 to 7 sugars carbons which are: ribulose 5-phospate, ribulose 5-epimerase, transketolase, and transadolase (26, 34, 37). These enzymes have been studied extensively and their function characterized in *E coli* mutants (34, 38). Members of Thermotogales such as *Tma* and *T. neapolitana* contains all these enzymes due to its ability to utilize pure pentoses and from plant material (21, 39-41).

Homologous Recombination:

Genetic recombination is a coordinated mechanism in which genetic material is cleaved and joined to other genetic materials (42). This process is initiated by DNA double strand breaks (DSBs) due to endogenous and exogenous sources (43, 44). For cell survival, it is imperative that bacteria utilize pathways that fix these breaks in turn helps maintain genomic stability (43).

Homologous recombination (HR) and non-homologous end joining (NHEJ) are pathways that repair DSBs. HR is used to synthesize a new DNA molecule by recombining of damaged DNA combining with an undamaged DNA in which there is equence homology. In NHEJ pathway little or no sequence homology is required for recombination to take place. Even though the activation of these pathways depends upon different factors in an organism, the components of each pathway are linked between all three domains of life (45-47). Furthermore, there has been extensive genetic and biochemical analysis, mainly in *E.coli*, that has led to the identification and function of each recombination enzyme in the HR pathway (48-50). However, a comparative genomic approach shows that some genomes are missing presynaptic enzymes involved in the HR process (45, 51). *Thermotoga maritima (Tma)* is a deeply rooted (Fig 1.1) hyperthermophilic bacterium that

lacks several key bacterial recombination enzymes (Table 1.1; Table 1.3; Table 1.7) (1, 6, 22, 52). Analysis of its genome shows it contains a greater portion of archaeal genes (7-11%) than any of the other bacterial genomes and may have gained archaeal recombination enzymes. (Table 1.2) In fact, it has been reported that the *mre*11 recombination enzyme, a mammalian protein that function in DNA repair, has been purified and characterized from *Tma* and archaeal species (13, 53-59).

Genetic Systems in Hyperthermophiles:

Thermo-instability of antibiotic at high temperatures has made it challenging to develop genetic system in hyperthermophilic micro-organisms. (Table 1.5) However, there has been a few genetic markers that have been established in other hyperthermophilic microorganism. *Thermus* (Bacteria) and *Sulfolobous* (Archaea) species are two of the best-studied organism for which genetic marker have been developed (60, 61). *Thermus* spp can thrive at temperatures above 75^{O}C and a study shows it is essentially competent in every growth phase demonstrating a unique system to be utilized for genetic research (60). With no appropriate selectable marker established the introduction of selectable markers such the tryptophan synthethase gene *trp*B was cloned on a plasmid and transformed into *T. thermophilus* by natural competence (62). Other plasmids such as the pTT8 that carries the thermostable kanamycin nucleotidyltransferase gene was developed to be used as selectable markers. The kanamycin resistant gene showed an increased in transformation efficiency when utilizing circular DNA (100-fold) as compared to the use of linear DNA (63, 64). Besides antibiotic used for genetic markers in *Thermus* spp, disruptions of genes that are associated with the uracil biosynthesis pathway have been reported in eurkaryotes. Deficiency mutants in this pathway, confers resistant to the 5-Fluroorotic acid, thus the means to use *pyr*E as a selectable marker (65). A study shows the development of *pyr*E as

a selectable marker by taking advantage of natural competency in the *pyr*E mutant for transformed with the *T.flavus* cosmid and characterized by gene mapping (66). This method was also used for the closely related *Thermus thermophilis* to develop a selectable marker by isolated of uracil auxotroph, cloning, and analyze of the *pyr*E gene (67).

The first evidence of recombination in an archaeal genome was observed in *S. acidocaldarius*. (68) The rare occurrences led to new experimental possibilities for genetic studies of hyperthermophiles (68). However, evidence of genetic transformation and isolations of viruses has made *Sulfolobous solfatarius* the model organism for genetic studies for the investigation of biological process in hypherthermophilic crenarchaeota. (69) There are numerous shuttle vectors and genetic markers that have different features for the manipulation of *Sulfolobus* spp (61). One example is the complementation of the defected *pyr*E genes by using the pMJ03 that contains pUC18, the *pyr*E/F gene, and a reporter gene, *lac*S. Disruption studies have also been evaluated in *S. solfatarius*. The *mer*A gene encodes for the resistant to mercury, disruption was investigated by placing the *lac*S gene in between the *mer*A gene that eventually lead to a reduction in growth when challenged with mercury chloride (70). This strategy was also used to evaluate the role of *a*-amylase in the presents of starch. The *lac*S gene was used as the genetic marker for alteration of the α-amylase gene which was characterized by observation of no zone of clearance on solid medium as compared wild type to measure its activity (71). These genetic methods have made it easy to conduct genetic studies in *S. solfataricus* with the need to develop more basic genetic tools to improve genetic system (61). Recently, a genetic system has been developed for *Pyrococcus furious* in which they were able to

utilize a shuttle vector and conjugation as a means to introduce DNA to make knockouts(72, 73).

There has been extensive efforts for developing genetic tools to study *T. maritima* and *T. neapolitana* (74), (75), (23), (76), (77). These studies provided defined growth media (78), reproducible plating methods, the means to obtain auxotrophic mutants (78), the discovery of the first plasmid in this lineage (74) and methods to introduce DNA into *T. maritima* using a vector constructed from the native plasmid (77). The latter methods gave very low transformation frequencies and we could not demonstrate that our plasmid was maintained in colonies growing on plates, thus limiting its utility. Low frequency transformation using a standard $CaCl_2$ transformation protocol like that used to transform *Thermococcus kodakaraensis* (79) (77). Previous methods were likely more limited by thermal lability of the antibiotic selective agents and plasmid instability than by the initial transformation efficiencies (77). Future efforts will use stable chromosome-encoded markers.

While a genetic system for Thermotogales has not yet been developed, there are established genetic systems for other hyperthermophilic microbes. These past accomplishments provide a strong basis for the proposed studies by providing successful genetic strategies that can be applied to Thermotogales and because they provide new routes for protein analysis. Genetic systems are available for several hyperthermophilic genera including *Sulfolobus* (81). Since structure function studies on thermophilic proteins are often confounded by an inability to produce recombinant protein in mesophilic hosts like *E. coli*, the genetic systems of *Sulfolobus* and *Metallosphaera* provide important alternatives; heterologous expression of Thermotogales genes in *Sulfolobus* or

Metallosphaera hosts can provide recombinant protein. Like Thermotogales, *Sulfolobus* and *Metallosphaera* are thermophiles but they belong to the Phylum Crenarchaeota within the Domain Archaea while Thermotogales belongs to the Phylum Thermotogae within the Domain Bacteria. Interestingly, all these organisms share phylogenetic topologies comprised of deeply branching lineages that originate close to the main root of prokaryotic taxa. The crenarchaeotal taxa (*Sulfolobus* and *Metallosphaera*) include both aerobes and anaerobes that grow in liquid and on solid media and have sequenced genomes (80) (81). The development of genetic methods for *Sulfolobus solfataricus* began with mutant isolation (82). The continued absence of plasmid-based vectors led to successful efforts at directed chromosome recombination (71); this may have particular relevance to Thermotogales where plasmids have not been successful. In *Sulfolobus*, these functional genomic methods provided direct identification of gene identities despite availability of genome sequence data (71). The development of a non-reverting selection strain improved the chromosomal recombination method by increasing recovery of targeted recombinants (83). Further developments included methods for creating integrated promoter gene fusions (84). These methods have been compiled and led to collaborative (85) (86) and independent use of these genetic methods (87) (87) (88). These successes support our contention that similar methods will be developed for Thermotogales.

Competency in Bacteria:

Horizontal gene transfer (HGT) is defined as the uptake and maintenance of extracellular DNA from one species to another via conjugation, transduction, or transformation (42, 89-91). These three mechanisms enable bacteria to acquired new traits that allow the bacteria to adapt to their ever changing environment. (92) Even though all

three methods successfully incorporate exogenous DNA into bacteria chromosome or retention of autonomously replicative plasmids, only the natural transformation mechanism is initiated by the donor cell. (91) These transformable cells contains all require proteins that makes this process possible however conditions must be suitable for activation of these genes. (42, 48, 93) This process is called competency. There are many gram positive and gram negative bacteria that are naturally competent in which the genes that encoded for uptake of exogenous DNA naturally have been studied and characterized (94-99). For this process to occur the following three mechanism must be activated: (i) genes that encodes the function for transfer of circular or linear DNA across the cell membrane via channels; (ii) the transport of these DNA molecule through this channel; and (iii) genes required for the process of and incorporation into the recipient cell via homologous recombination. (100).

Lateral gene transfer (LGT) plays a crucial role in evolution while creating genetic diversity. Although it was believed that LGT is rare in bacteria, it is frequent enough to allow rapid acquisition of novel functions needed to promote adaptation and speciation. Bacteria are promiscuous and homologous recombination occurs even between bacteria that have as much as 25% sequence divergence (5). Genome-wide analysis reveal that at least 3% of genes of most free living bacteria have been acquired by interdomain (e.g., Archaea to bacteria) horizontal transfer (1). Recombinational events in bacteria are capable of relaxing the diversity purging effects of periodic selection and preserve the genetic diversity. Recombination between different strains and species of bacteria plays a crucial role as a source of genetic variation. Increasing rates of adaptive evolution with the help of recombinational events can provide a selective advantage over identical and fit populations

that are unable to engage in horizontal gene transfer. An important criterion for identifying species clusters of evolutionary significance is to find characteristic features (ecological or genetic) that distinguish one phylotype from its close relatives (3). *Tma* contains 7-11% of archaeal genes in its chromosome indicating the presence of a competency system that takesup extracelluar DNA from its environment. (101) Even though *Tma* does not contain all of the machinery required for DNA uptake, it has competency genes homologous to *H. influenzae* and *B. subtilis* (7). The competence genes homologous in *Tma* from *H. influenza* are *comE*, *comM*, and the DNA processing chain A (*dprA*) and from *B. subtilis comEA* and *comFC*. (7) These identified com genes in *Tma* may play a role in the uptake and transport of DNA across the "toga" or periplasm while the *dprA* plays a role in the nicking and resection of the incoming DNA. (7) Recombination between regions of DNA with similar sequences is termed homologous or RecA-dependent recombination. (Table 1.7) Homologs of RecA have been found in almost all bacteria. There are two main families of RecA like proteins: the bacterial RecA family and the eukaryotic Rad51 like family. Rad51 also includes archaeal RadA subfamily (2). *Tma* has both *recA* and *radA* genes, which suggests that *radA* is transferred from an archaeal origin. (7)

A surprising correlation exists between the similarities in the lifestyles of very distantly related organisms (bacterial and archaeal hyperthermophiles) and the extent /rate of LGT between them. This raises an interesting question about the role of LGT, whether it is adaptive or opportunistic in the organism's niche. Different methods are available to infer past horizontal transfer events. Whole genome comparison is one method which strongly suggests that LGT events involving prokaryotes have been more substantial than previously imagined. This and other phylogenetically based methods can only identify the

genes that probably have entered a particular genome by LGT. A complete understanding of the biological significance of LGT will require experimental analysis of the genes involved in LGT in order to determine if and how this bacterium regulates acquisition/loss of the DNA, including the genes detected as acquired by the bioinformatics analyses seems particularly appropriate for addressing the evolutionary questions.

Microbial Iron Reduction:

Iron (the most abundant element on Earth) makes up the majority of subsurface and aquatic environments making it the most readily used terminal acceptors (102-104) (105). It is well know that microbes from both Archaea and Bacteria lineages have the ability to utilize Fe(III) as an electron acceptors, thus controlling the iron redox balance in most environments (105, 106). Fe(III) reduction is an energetically plausible reaction than sulfate reduction, however, generates minimal free energy (107). This process is called dissimilatory Fe(III) reduction and specified by iron being using as an electron acceptor when bacteria undergo respiration. Furthermore, one of the hallmark indication of dissimilatory reduction is the accumulation of Fe(II) during bacterial respiration. However, it has been reported that acetate is the main source for Fe(III) reduction upon fermentation by *Geobacter metallireducens* (complete oxidization of acetate) and *Shewanella* putrefaciens (108, 109). These are the two best known organisms that has been studied extensively for iron reduction. They are recognized for their ability to couple the oxidation of organic compounds by reducing Fe(III) (109). Even-though the mode of action (dissmilatory reduction) has been defined, the mechanism is poorly understood. There are three know strategies that most iron reducing bacteria may employ (i) direct contain of the microbe with substrate to delegate electrons to it, or (ii) utilize soluble iron as a mean to

transfer electron via transport chain, or use an intermediate (shuttling) or manufacturer its own (106). It is know that Fe(III) reducing is mainly done by Proteobacteria, however there has been evidence of bacterial respiration phylogenetically distinct from Proteobacteria. To date, all hyperthermophilic organism examined can conduct Fe(III) reducing. These organisms warrant further study to understand their impact in various sedimentary habitats (105).

The investigation of microbial Fe(III) reduction by hyperthermophilic organism is of relevance because they are closely related to the last common ancestors. Furthermore, this form of respiration (dissimilatory reduction) may have a profound environmental and evolutionary impact (110). The phylogentic analysis shows that there are a variety of microbes that conduct dissimilatory reduction of Fe(III) throughout the Archaea and Bacteria domain that can thrive in extreme environments. These temperatures range from 20^{O}C- 121^{O}C (106). However, little is known about microbial iron reduction at high temperatures (111). In studies conducted by (111) on isolates closely related to *Thermoanaerobacter ethanolicus*, they were able to show that 3 isolates had the ability to reduce a wide range of metals with acetate, hydrogen (only one of the isolates), and lactate as the electron donors. Furthermore, by adding metabolic inhibitors (that block function of enzymes such hydrogenase and ferric reductase) it inhibited the cultures ability to reduce Fe(III). This suggests that the reduction of the various metals could be linked to an enzymatic function. These isolates and other thermophilic isolates that have been identified such as *Thermus* spp. grow around 65^{O}C. However, there have been studies on hyperthermophilic organism such as *Thermotoga maritima* and *Pyrobaculum islandicum* that can grow above 65^{O}C. It has been reported that there was an 10-fold increase of

Pyrobaculum islandicum when cultured in medium containing hydrogen (electron donor) and Fe(III)-citrate as the electron acceptor. Moreover, *P. islandicum* was also able to reduced toxic metals such as uranium and chromium (110). Recently, a study showed that *P. islandicum* reduces Fe(III) citrate via a different mechanism that does not seem to be c-type cytochrome dependent. This was determined by the increase activities in NADH-dependent ferric reductase enzyme when cells were cultures in the presents of Fe(III). This mechanism suggest that cells do not have to come into contain with insoluble iron oxide, which is unlike what occurs in *Shewanella* and *Geobacter* species (112{Feinberg, 2008 #332, 113). As described above, the first bacteria identified that could conduct respiration were fermentative microbes. One of these hyperthermophilic bacteria that has shown promise as a Fe(III) reducer is *Thermotoga maritima*. A study conducted by (114) shows *Tma* exhibiting Fe(III) reduction by growing in medium with H_2 as the electron donor and Fe(III) citrate as the electron acceptor and insoluble iron. This suggests that *Tma* is able to grow not only by fermenting carbohydrates but by respiration and transfer electrons to a solid surface via an electron shuttle (115). However, there has not be much effort since to understand the mechanism required for Fe(III) reduction by *Tma*. With a sequenced genome, our understanding of Fe(III) reduction by other hyperthermophilic organism, and an established genetic system in *Tma*, it would make a preferable model organism for the analysis of Fe(III) reduction (7). These findings described above warrant further study because it expands our knowledge about the temperature relatively to iron reduction (mesophiles) at which dissimilatory reduction occurs and it importance on understanding metal geochemistry (116). Furthermore, it may give us insight on how Fe(III) reduction

may have played an intricate role on early Earth as well as their contribution to the modern day hot biospheres (114).

Goal of this study:

The study of hydrogen production along with soluble and insoluble iron reduction in mesophilic and hyperthermophilic archaea has been done (114). Since *T. maritima* is a hyperthermophilic bacterium and excretes 4 mol of H_2 via removal of excess reductants this makes it suitable for the investigation of how it produce hydrogen and the mechanism required for the reduction of iron. The lack of a genetic method for creating targeted mutations has resulted in inability to quantitatively measure the in vivo H_2 yields by pathway manipulations. Despite receiving considerable efforts to advance the genetic methods for *T. maritima,* manipulation of the chromosome has remained elusive, hindering its use as a model hyperthermophile. *T. maritima* is capable of fermenting a broad range of sugars to form acetate, carbon dioxide, traces of lactate and near theoretic yields of molecular hydrogen. To facilitate this, a homologous chromosomal recombination method has been developed to inactivate genes to better understand the significant contribution of sugar fermentation towards H_2 formation as well as its role in the reduction of soluble and insoluble iron. The study will explore the following questions:

i. Does the inactivation of genes that play a role in fermentation alter the production of hydrogen formation?

ii. Does *T. maritima* metabolism shift when cultivated with an alternative electron sink?

iii. What genes are involved in soluble and insoluble iron reduction? Does this process occur in a sugar dependent manner?

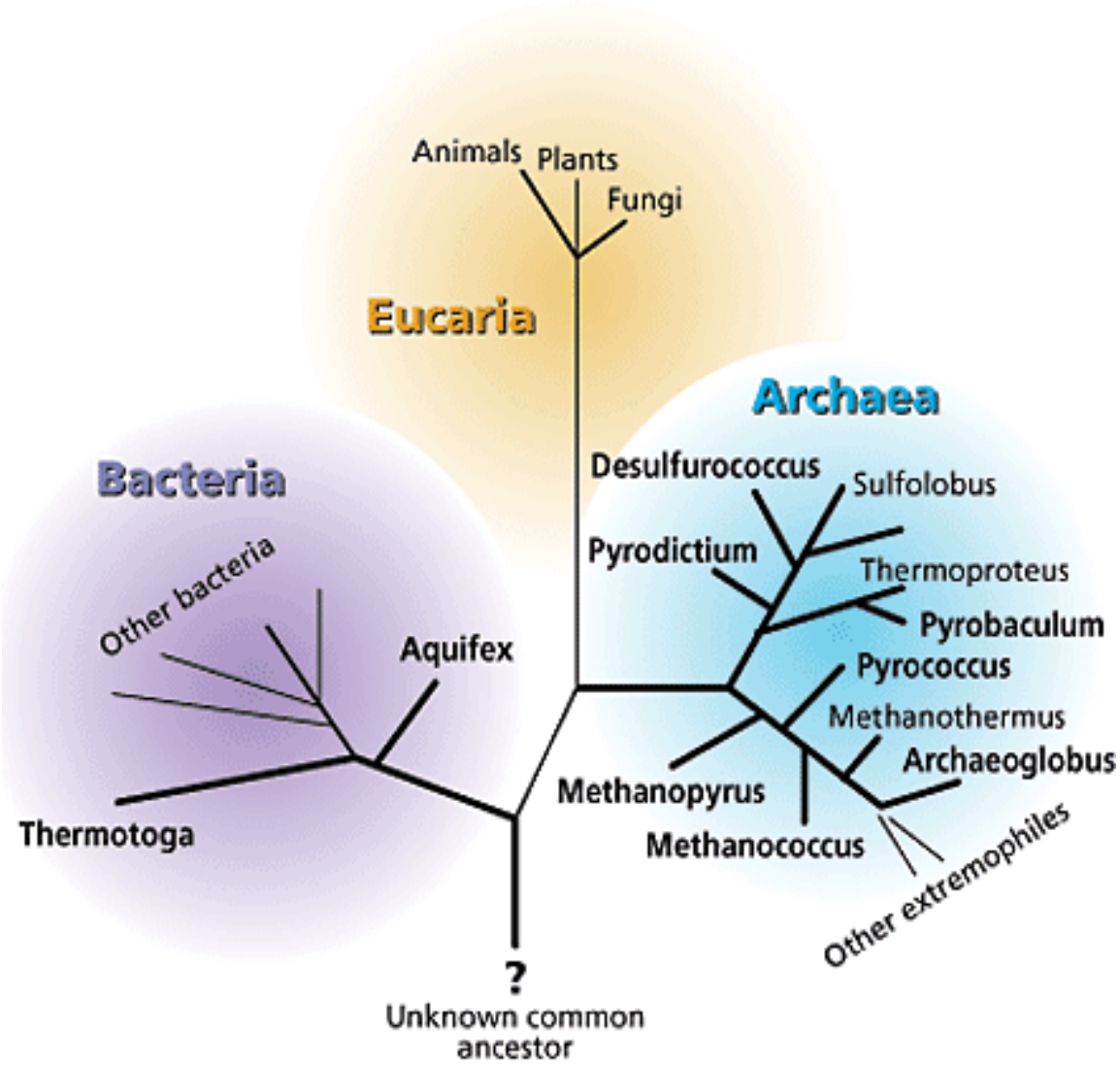

Figure 1.1: Phylogenetic tree of the three domains of life. Bacteria, Archaea, and Eukarya represent the three domains of life.

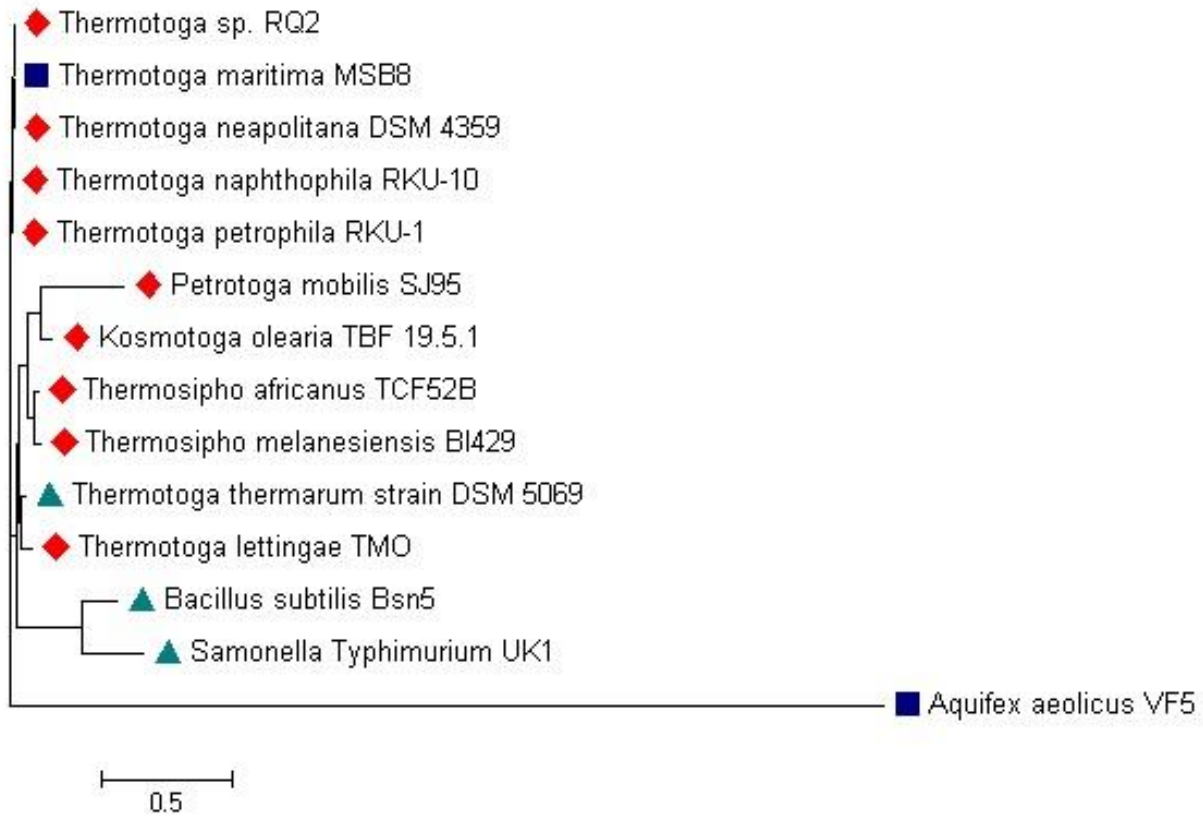

Figure 1.2: MEGA phylogenetic tree of Thermotogales. The tree is a representation of the closely and distant related *Thermotoga* species.

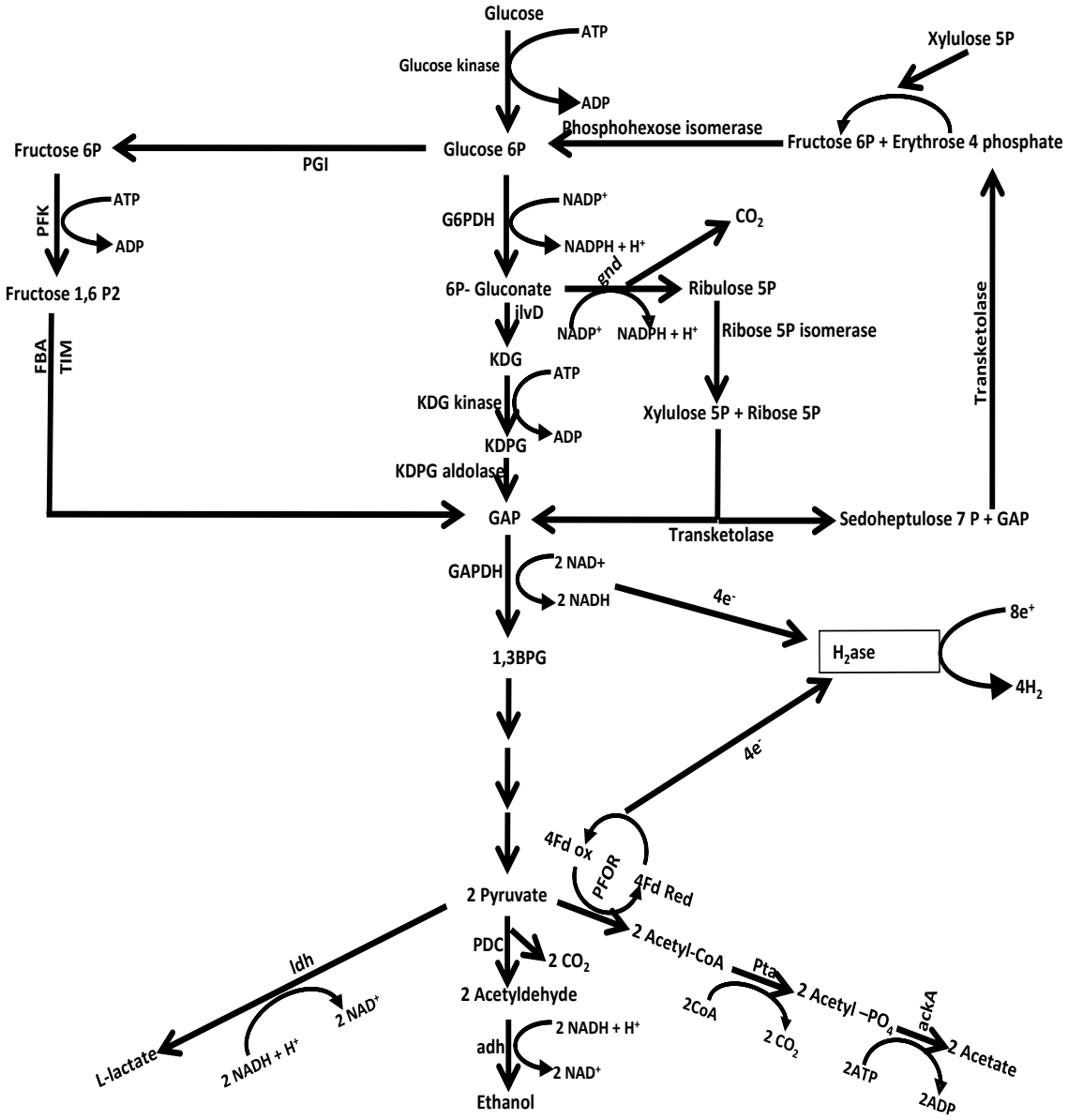

Figure 1.3: Schematic representation of carbon flow during fermentation using EMP, ED, and pentose phosphate pathway in *T. maritma*.

Table 1.1: Bacterial Enzymes that play role in DNA metabolism in *Thermotoga maritima*

Gene name	ORF	Location	AA Length	Pathway
recA	TM1859	1842056..1843126	356	Homologous recombination
Repair endonuclease	TM0382	402170..401417	232	Base pair excision
Endonuclease III	TM0366	384715..385356	213	Base pair excision
Endonuclease V	TM1865	1846667..1847344	225	Base pair excision
DNA mismatch repair protein (*mut*S)	TM1719	1695366..1697747	793	Mismatch repair
DNA mismatch repair protein (*mut*L)	TM0022	19348..17798	517	Mismatch repair
DNA mismatch repair protein, putative (Bs)	TM1278	1306700..1304427	757	Mismatch repair
Excinuclease ABC subunit A(*uvr*A)[Aa]	TM0480	509595..506845	917	Nucleotide excision repair
Excinuclease ABC subunit B(*uvr*B)[Mta]	TM1761	1738560..1736566	667	Nucleotide excision repair
Excinuclease ABC subunit C(*uvr*C)[Mta]	TM0265	275224..276897	557	Nucleotide excision repair
Holliday junction (*ruv*A)	TM0165	171423..170848	192	Homologous recombination
Holliday junction (*ruv*B)	TM1730	1706619..1707623	334	Homologous recombination
chromosome segregation SMC protein	TM1182	1198722..1194156	1170	DNA replication

Table 1.1 continued

Holliday junction (*ruv*C)	TM0575	605353..606011	169	Homologous recombination
DNA topoisomerase	TM0258	266645..269116	633	DNA replication
DNA polyermase III, alpha sub(*dna*E) [Bs]	TM0576	605307..610640	1337	DNA replication
DNA polyermase III, alpha sub, put [Aa]	TM0461	492139..488853	843	DNA replication
DNA polyermase III beta sub(dnaN) [Hi]	TM0262	271392..272822	370	DNA replication
DNA polyermase III epsilon sub, put [Aa]	TM0496	525146..524407	190	DNA replication
DNA polyermase III gamma/tau sub (dnazX)	TM0686	711223..713089	480	DNA replication
DNA gyrase, subunit A	TM1084	1100654..1097516	807	DNA replication
DNA gyrase, subunit B	TM0833	857372..859856	637	DNA replication

Table 1.2: Eukaryotic Enzymes in DNA metabolism in *Thermotoga maritima*

Gene name	ORF	Location	AA Length	Pathway
radA	TM0199	213145..214467	440	Homologous recombination
radC	TM1557	1549286..1549954	222	Homologous recombination
*mre*11	TM1635	1622413..1623570	387	Homologous recombination
Reverse gyrase	TM0173	178469..182777	1107	DNA replication

Table 1.3: Enzymes that play a role in DNA metabolism not found in *T. maritima*

Pathway	Gene	Bacteria/Eukaryote
Mismatch repair	*uvr*D	Bacteria
Mismatch repair	*mut*H/*mlh*1/*mlh*2	Bacteria/ Eukaryote/ Eukaryote
Homologous recombination	*rec*BCD/*rec*FOR/RAD52	Bacteria
Homologous recombination	*nsb*1	Eukaryote
DNA polymerase	*umu*C/*din*B	Bacteria

Table 1.4: Comparative analysis of H_2 production in thermophiles, hyperthermophiles and Archaea

Organisms	Domain	Growth Temp.	Culturing Condition	Substrate	End Products	H_2/hexoses	References
T. maritima	B	80	Batch	Glucose	Acetate, lactate	4	(52)
T. neapolitana	B	80	Batch	Glucose	Acetate, lactate	2.4	(117)
T. elfii	B	65	Controlled batch	Sucrose	acetate	3.3	(118)
C. sachharolyticus	B	70	Controlled batch	Sucrose	Acetate, lactate	3.3	(2, 118)
T. tengcongensis	B	75	Batch	Starch	Acetate, ethanol	2.8	(119)
T. sachharolyticum	B	55	Batch	Cellobiose	Acetate, lactate, ethanol	0.87	(120)
C. thermocellum	B	60	Chemostat	Cellulose	Acetate, formate, ethanol	1.65	(121)
T. kodakarensis	A	85	Chemostat	Starch	Acetate, alanine	3.3	(122)
P. furiosus	A	90	Chemostat	Maltose	Acetate, butyrate	2.9	(123)

Table 1.5: Developed Genetic Systems in Hyperthermophiles

Organism name	**Cloning vector**	**Developed Genetic marker**	**Outcome (+/-)**	**Reference**
Thermus spp (T. thermophilus & T. flavus)	pVUF10, pVUF10.5 & pVUF10.5.9	*pyrE* and *pyrF*, Kanamycin nucleotidyltransferase	Positive	(65, 66)
Thermococcus kodakaraensis KOD1	pSY2	*pyrE* and *pyrF*, HMG-CoA reductase gene	Positive	(124)
Pyrococcus furious	pGLW027	*pyrF*	Positive	(72)
S. sulfataricus and S. acidocaldarius	pAmy2	*pyrF* *lacS*	Positive	(68, 71)
C. bescii	pSC101 pJMC010	*pyrF* Kanamycin nucleotidyltransferase	Positive	(125, 126)
Thermotoga neapolitana	None	*pyrE* mutant	Negative	(76)
Thermotoga maritima MSB8	pJY2	Km^r gene clone into pBT, then RQ7 cloned into pJY; Amp^r,Km^r	Negative	(77)
Thermotoga RQ7	pDH10	Kan resistance gene	Negative	(127, 128)
Thermotoga RQ7	pHX02, pHX04, and pHX07	Kan resistance gene	Negative	(129)

Table 1.6: Assessment of *T. maritima* promoter strength via transcript abundance analysis

Target gene	**Avg. C_T values**	**Reference gene (rpoD), C_T values**	**ΔC_T (Avg. target gene C_T - avg. rpoD C_T)**	**$\Delta\Delta C_T$**	**Normalized to *pilA* gene $2^{-\Delta\Delta CT}$**
pilA	16.88 ± 0.99	14.19 ± 0.70	2.69 ± 0.29	0	1
groES	16.90 ± 0.79	14.19 ± 0.70	2.71 ± 0.086	0.67 ± 0.20	0.75
hisI	20.44 ± 0.01	14.19 ± 0.70	6.25 14.19 ± 0.69	3.56 ± 0.40	0.09
ldh	19.96 ± 0.12	14.19 ± 0.70	5.76 ± 0.57	3.07 ± 0.29	0.12

The *pilA* promoter was found to be 1.6, 11.48 and 8.4 fold stronger than the *groES*, *his-I* and *ldh* promoters, respectively. These data provide a basis for appropriate promoter selection for transgene expression in *T. maritima*.

DNA sequence of codon optimized *T africanus* fused to *Tma groESp*

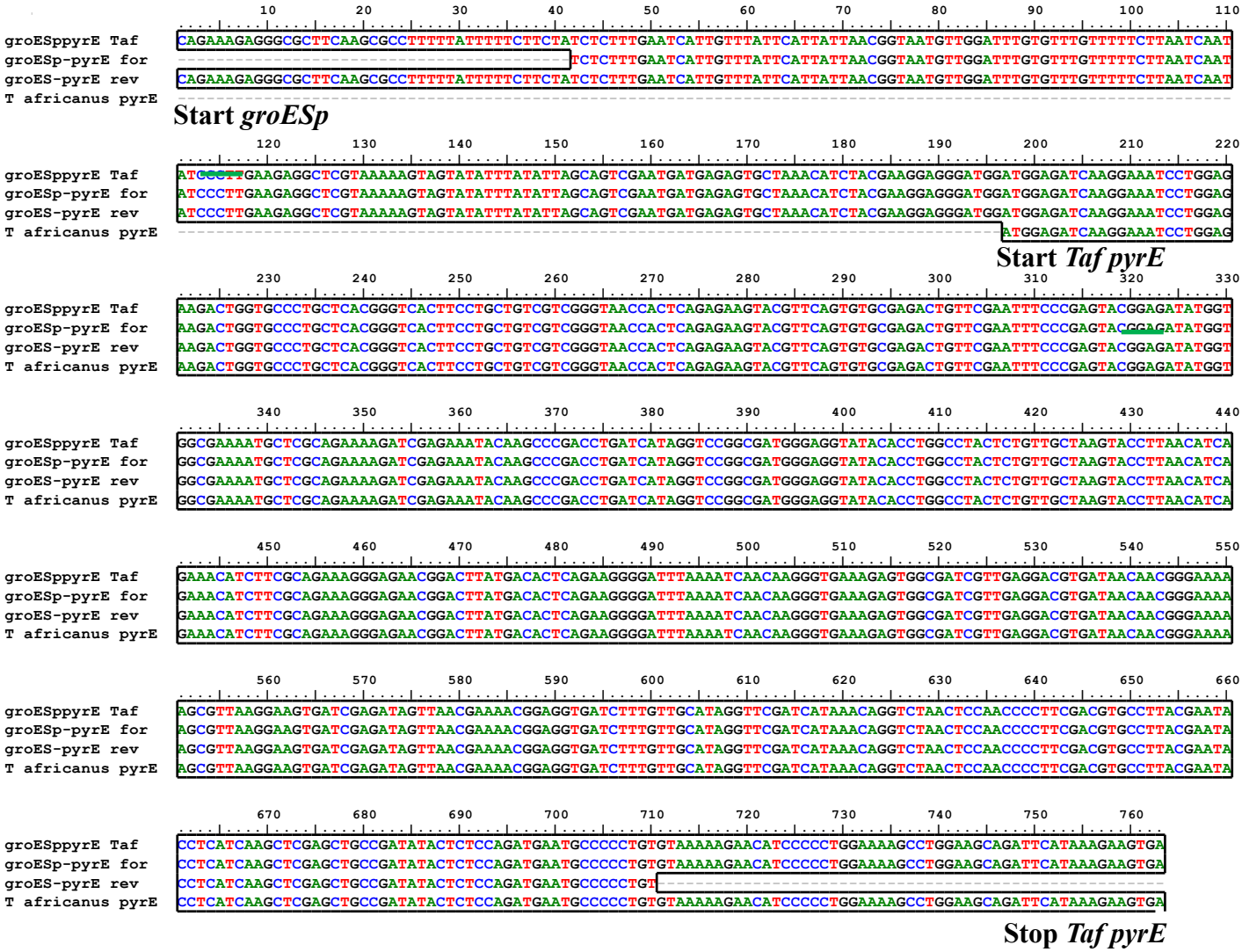

Table 1.7: Recombination enzymes found in the three domains of life

	Unwinding of DNA	Nuclease Activity	Recombinase Activity	
Species				
T. maritima	topA	Mre11	RuvA/B	RuvC
E coli		recBCD/sbcC/D	RuvA/B	RuvC
S. cerevisae	Spo11	Mre11/RAD50	Rad54	CceI
Archaea	Spo11	RADA/RADB/RAD54	Rad54	Hjc/Hje

CHAPTER 2

Contribution of Pentose Catabolism to Molecular Hydrogen Formation by Targeted Disruption of Arabinose Isomerase (*araA*) in the Hyperthermophilic Bacterium, *Thermotoga maritima*.

1. Abstract

Thermotoga maritima ferments a broad range of sugars to form acetate, carbon dioxide, traces of lactate and near theoretic yields of molecular hydrogen (H_2). In this organism, the catabolism of pentose sugars such as xylose or arabinose depends on the interaction between the pentose phosphate pathway, the Embden Myerhoff pathway and the Entner Doudoroff pathway. While values for H_2 yields have been determined using pentose-supplemented complex media (21) and predicted by metabolic pathway reconstruction, quantitative *in vivo* measurements derived from pathway elimination have not been reported reflecting the lack of a genetic method for the creation of targeted mutations. Here, a spontaneous and genetically stable *pyrE* (orotate phosphoribosyltransferase) deletion mutant was isolated and used as a recipient to refine transformation methods for its repair by homologous recombination. To verify the occurrence of recombination and to assess the frequency of crossover events flanking the deleted region, a synthetic *pyrE* allele was employed encoding synonymous nucleotide substitutions. Targeted inactivation of *araA* (arabinose isomerase) was accomplished using the *pyrE* deletion strain as a recipient with a divergent and codon optimized *T. africanus pyrE* transcriptionally fused to the native *T. maritima groESL promoter (groESp*). Mutants

lacking *araA* were unable to catabolize arabinose in a defined medium. H_2 synthesis using arabinose supplemented complex medium was compared between wild type and *araA* mutant cell lines. The difference between strains provided a direct measurement of H_2 production that was dependent on arabinose consumption. Development of a targeted recombination system for manipulation of *T. maritima* provides a new strategy for addressing basic and applied studies involving H_2 formation and life at temperature extremes in the bacterial domain.

2. Introduction

Thermotoga maritima is a hyperthermophilic anaerobic bacterium that ferments simple sugars to H_2, acetate, lactate and carbon dioxide (6). In complex medium it grows optimally at 80ºC with a generation time of 75 minutes (6). Because of its rapid growth, and aerotolerance it was the recipient of considerable investigative effort. This included genome sequencing (7, 18, 130, 131), comprehensive functional genomics for protein structural characterization (18, 132), and transcriptomic studies using oligonucleotide arrays (5, 9-14, 16, 17). However, in the absence of a genetic system for genome manipulation, its use as a model hyperthermophile has slowed.

There have been advances in genetic methods for *T. maritima*. Marker selection strategies used the analog 2-deoxy glucose to recover resistance mutants in an effort to use sugar utilization genes as a marker (133). Spheroplast-based transformation that removed the proteinaceous toga to promote DNA uptake using liposomes (77). And the use of replicating plasmids that carried heat stable antibiotic resistance for selection at elevated growth temperatures (77, 127-129). However despite these approaches, manipulation of the chromosome has remained elusive.

The impact of pentose metabolism on H_2 synthesis is crucial for biohydrogensis applications using a fermentative organsim such as *T. maritima*. This is because a significant component of lignocellulosic plant feedstocks are made of 5-carbon sugars such as xylose and arabinose. These sugars are metabolized via the pentose phosphate pathway (PPP) that consists of oxidative and non-oxidative components. The main function of the non-oxidative pathway is to generate C3through C7 sugars from 5-ribose phosphate that can be used for nucleic synthesis and that enter the oxidative pentose phosphate pathway and in turn glycolysis for production of ATP and reductant. Pentose metabolism only recently has been studied in *Thermotoga* species where a H_2 yield of 3.33 moles per mole of sugar was reported (21). To better understand the contribution of arabinose to H_2 formation through pentose catabolism, a method for homologous chromosomal recombination was developed and used to inactivate catabolism of this sugar by targeted mutation of *araA*, the first step in arabinose catabolism.

3. Materials and Methods

3.1 Strains and cultivation. Unless otherwise indicated, *T.maritima* MSB8 was cultivated at 80ºC under anaerobic conditions. Complex medium (CM) was prepared as previous described by (134). A solid medium was prepared using 6g/L of gellan gum (Sigma-Aldrich). Cultivation routinely used 10 mL volumes of medium in Hungate tubes supplemented by addition of 100 μL of a 10% sterile stock solution of Na_2S, 40μl of a sterile stock solution of 10% KH_2PO_4, and 500μl of a sterile stock solution of 10% (w/v) maltose. Tubes were then sealed with butyl rubber stoppers (Bellco Biotechnology), crimped with a metal band, and the headspace removed by N_2 sparging. The anaerobic

culture tube with growth medium was inoculated by adding cells from a frozen permanent into 300µl of complex medium in a sterile 1.5ml Eppendorf, medium collected by a 1cc syringe attached to a 20 ½ G Needle then added into complex medium tube. The Hungate tube is incubated at 80°C for 18-24hrs to reach mid-log phase.

A defined medium was prepared as previously described in (134) with the following modification per liter: Removal of tryptone and yeast. The pH of defined medium was adjusted to 7 before autoclaving by adding 20µl of 100% H_2SO_4. Wild type (WT)*Tma* was cultivated in 15ml defined medium of which 750µl of 10% Maltose, 750µl of 10% NH_4CL, 150µl of 10% Na_2S, 180µl of 10% KH_2PO_4, 50µl of Trace Elements, 15µl of Wolfe's vitamins, and 375µl of a 2mg/ml 20 amino acid stock solution. Headspace was removed with N_2. Wolfe vitamins was prepared as previously described in (135). A final concentration of 50µg/ml of uracil was added to defined medium to evaluate auxotrophy. For the preparation of defined medium plates, 6g of phytagel (Sigma Aldrich-P8169) was added to 1L and a final concentration of 400µg/ml of 20 amino acid solution was added.

3.2 Uracil auxotroph isolation. For the isolation of *T. maritima* uracil auxotroph was done as previously described by (76) with modifications. Cells were plated onto CM plates and incubated at 80ºC in an Almore Anaeorbic jar with an EZ Gaspak for 2 days to get single colonies. 10 independent individual isolates were picked from the plate and inoculated into 10ml of complex medium in Hungate tubes. For isolation of 5-FOA resistant mutants, 100µl of mid-log phase cells from each independent individual culture was top spread onto 400µg/ml 5-FOA and 50µg/ml uracil drug plates and incubated in an anaerobic jar at 80ºC for 2 days. A single colony from each plate was cultured in

complex medium, genomic DNA isolated, and the *pyr*E allele was amplified. Uracil auxotrophy was evaluated by cultivating 5-FOA resistant mutants in defined medium with and without uracil supplementation (50μg/ml).

3.3 *T. maritima* transformation. Two types of transformation were used in this study, transformation using electroporation and natural transformation. The electroporation procedure used spheroplasts. For the preparation of spheroplasts, 50 mL cultures were processed as described by (77) with modifications. One μg of plasmid pB1183 was added to 50μl of spheroplasts ($1x10^8$ cells/mL) and electroporated using a Bio-Rad Gene Pulser at 1.8kV and 200 ohms and inoculated into 10mL of complex medium for a recovery period of 24hours. Cells were recovered by centrifugation, washed twice with equal volumes of defined medium and 0.1 mL of the suspension was inoculated into selective media. Inoculated cultures were incubated at 80ºC for 2-3 days and transferred to selective solid medium. Individual colonies were inoculated into complex medium and the cultures used for DNA analysis. For the preparation of cells for natural transformation cells were processed as described (128) with the following modifications. Recovered cells were resuspended in 1ml 20mM Sucrose and incubated at 60 ºC for 20 minutes. A volume containing $1x10^8$ cells was transferred to a 1.5mL microfuge tube and 1.5μg of DNA was added and the mixture incubated for 30 min a 55 ºC followed by inoculation into 10 mL of selective medium and incubation at 80°C for 18 hours. Cells were then collected by centrifugation, resuspended in 1mL of defined medium and adjusted to a cell density of 10^9 cells/mL. 10^8 cells were inoculated into selective medium to recover recombinants.

3.4 Plasmid and strain construction. Repair of *pyrE-129* employed native and synonymous alleles of *pyrE*. The native allele varied in length including fragments of 1,120, 764, and 564bp in length (see Results). A 977bp synthetic *pyr*E allele contained two synonymous codon changes flanking he *pyrE*-129 deletion. All DNAs were cloned into pUC19 at *Sph*I and/or *Pst*I restriction sites and all cloned fragments were sequenced to verify composition. The *araA* disruption mutant was constructed by integration of the *groES*p::*pyrET*$_{Af}$ construct flanked by *araA* sequences at the chromosomal *araA* locus using the *pyrE*-129 mutant as recipient and selecting for uracil prototrophy. A codon optimized *T. africanus pyrE* allele fused at chromosomal position 516, 680 to *Tma groES*p at chromosomal position 532,233 and cloned into the *Bam*HI site of pUC19. This insert was then flanked by insertion of *araA* coding sequences. A 5' *araA* fragment (746bp sequence) was cloned at the pUC19 *Eco*R/*Kpn*I sites and a 3' *araA* fragment (745bp sequence) was cloned at the pUC19 *Sal*I/*Sph*I resulting in plasmid pPB1322 (**Table 2.1**). Strain PBL3004 was then transformed to uracil prototrophy using DM lacking added uracil. Liquid enrichments were plated onto DM plates and incubated anaerobically at 80 °C for three days. Genomic DNA was characterized for the *araA* allele by PCR using cultures prepared from colonies isolated from the selection plates cultivated in CM without selection for uracil prototrophy. *Tma* genome NC_000853.1 and *T. africanus* TCF52B (NC_011653.1) was used for generation of all primers and identification of all coordinates.

4. Results

4.1 Isolation and characterization of *T. maritima pyrE* mutants. Development of a genetic system to modify the *T. maritima* genome required a series of stepsincluding a genetic marker, marker-compatible media, specialized host strains, vectors,

transformation, and chromosomal recombination. A genetic marker that conferred pyrimidine prototrophy was developed through the isolation of spontaneous *pyrE* mutants. A defined solid medium was needed for clonal isolation of mutants, to determine their genetic stability, and to recover recombinants. A defined liquid medium was needed to verify strain phenotypes and in some cases to enrich for rare recombinants. While a defined liquid medium has been described (135), its use for a solid medium required addition of all standard amino acids (AA) to support colony formation. Uracil auxotrophs were recovered by the selection of isolates resistant to 5-fluoroorotic acid (5FOA) (76) using a solid medium at a frequency of 1×10^{-7} at an FOA concentration of 800 µg/mL. DNA sequence analysis of *pyr*E (TM0331) and *pyr*F (TM0330) from ten resistant isolates indicated all encoded loss of function mutations in *pyrE* while *pyrF* remained unaffected. These isolates all encoded the same *pyrE* mutation (*pyrE*-64) that consisted of a two nt deletion (-TG) at chromosomal positions 351,789 (-T) and 351,790 (-G), 155 nt from the 3'end of *pyrE*. This mutation resulted in a premature stop codon (TGA) 64 nt before the natural stop codon and therefore reduced protein length by 21 AA (from an original 187 AA). An auxotrophic phenotype for this isolate was confirmed by demonstrating that its growth in a defined medium was dependent upon uracil supplementation **(Figure 2.1)**. The *pyrE*-64 mutation reverted at a frequency of 1×10^{-7} as a result of a 2 nt insertion located 351,789 (T) and 351,790 (G), (*pyrE*-100). While *pyrE*-64 was relatively stable, a non-reverting mutation was pursued to improve the likelihood of recovery of recombinants. In this case, additional 5-FOA resistant mutants were isolated from ten independent cultures. PCR amplification of *pyrE* from one of these isolates produced a smaller amplicon consistent with deletion formation **(Figure**

3.1). DNA sequence analysis indicated it encoded a 129 nt deletion of *pyrE* located at the 5' end of the gene at nt 37 and that spanned chromosomal positions 351,419 - 351-548. This in-frame mutation was called *pyrE-129* and it reduced the length of orotate phosphoribosyl transferase by 42 amino acids. An auxotrophic phenotype was confirmed by demonstrating growth in a defined medium was dependent upon uracil supplementation and the mutant cell line was named PBL3004 **(Figure 3.1)**. Reversion analysis demonstrated *pyrE-129* was genetically stable with a reversion frequency of $< 1 \times 10^{-8}$ and therefore suitable for use as a recipient to develop targeted chromosomal recombination.

4.2 Homologous chromosomal recombination in *T. maritima*. A second required component for the genetic system was a properly designed recombinogenic DNA molecule. The initial design for this molecule considered both its length and topology. Linear DNAs and their circular forms included: 1128 bp spanning coordinates 350,919 - 352,048; 764 bp spanning coordinates 351,282 - 352,045; 564 bp spanning coordinates 351,382 - 351,945, and the *pyrE* ORF having 37bp 5' and 398bp 3' of the *pyrE-129* deletion (**Figure 2.2**). The 1128 bp molecule encoded 500 bp on both sides of the deletion, while the 764 bp allele encoded 137 bp of homology 5' to the deletion and 498 bp 3' to the deletion. Successful enrichments for prototrophic cells followed by formation of prototrophic colonies was observed only for circular forms of the 1128 and 764 bp molecules.

The final components for the genetic system was DNA transformation and chromosomal recombination. These two components however, could not be obtained

without an autonomously replicating vector. As such vectors have not been reported for *T. maritima* transformation and recombination were measured together arising from change in the allelic state of the *pyrE* genetic marker. An initial transformation procedure used spheroplasts as described previously (77). These cells lack at least portions of the toga (outer membrane and proteinaceous wall) and therefore are permeable to DNA. They are evident by their spherical morphology rather than normal elongated rod shape (6). The use of spheroplasts was replaced subsequently by the use of natural transformation as described previously for related *Thermotoga* species. However, for *T. maritima*, cell physiologic status was critical for obtaining recombinants. Using early mid-log, late mid-log, and early stationary phase cells for natural transformation, we were able to recover 200X more *pyrE*$^{+}$ recombinants as compared to using spheroplasts cells. The relative frequency of recombination using spheroplasts was $1x10^{4}$/μg of DNA while that for natural transformation was $5x10^{6}$/μg of DNA. In contrast to the longer DNA molecules, the shorter 564 bp molecule failed to produce recombinants using spheroplast or natural transformation despite repeated attempts that followed identical procedures. This suggested a more extended length of flanking homology was required for homologous recombination. Purified isolates were tested for prototrophic growth relative to controls and the *pyrE* locus was examined by PCR and DNA sequencing (**Figure 2.2**). All subsequent genetic crosses employed the natural transformation procedure.

4.3 Analysis of recombination using synthetic donor DNA. Occurrence of putative *pyrE* recombinants exhibiting a prototrophic phenotype might arise by contamination from wild type cells. To exclude this possibility, a synthetic *pyr*E allele was used to

repair uracil auxotrophy that encoded two synonymous substitution mutations located at chromosomal positions 531,385 and 531,694. These substitutions flanked the *pyrE-129* deletion and were positioned 27nt from the deletion end points. However, initial attempts to recover recombinants using this molecule failed. Because the synthetic DNA was shorter than the previously successful wild type molecules, longer versions of the synthetic DNA were tested that had extended chromosomal homology. One molecule had the addition of 300 bp 5' to the *pyrE*-129 deletion but did not produce recombinants. The other molecule included both the 5' extended region and an additional 530 bp 3' to the *pyrE-129* deletion. This symmetrically extended molecule produced recombinants. To assess the relative frequency of cross over events, ten independent isolates obtained from ten separate transformation reactions were recovered and analyzed. Of these, seven encoded both synonymous changes, and three encoded only one synonymous change all located 3' to the *pyrE*-129 deletion. (**Figure 2.3**) Because the addition of the 3' extended region enabled recovery of recombinants, this region was examined more closely for recombinogenic sequences. Interestingly the *Bacillus subtilis* Chi consensus sequence AGCGG was present and was located 351, 704 and 351,977 (upstream gene of *pyrE*) only at the 3'end of the *pyrE* allele. In *B. subitilis*, this Chi sequence is recognized by AddA and AddB site during recombination (136).

4.4 Construction and characterization of a divergent *T. maritima* selection marker.

Having demonstrated occurrence of homologous recombination at the *pyrE* locus, a genetic marker was developed that would allow targeted disruption of other *T. maritima* genes without interference from recombination at the native *pyrE* locus. Two components were required, a divergent *pyrE* lacking homology to the native *T. maritima pyrE* locus,

and a strong promoter to drive expression of the divergent *pyrE* allele. The divergent *pyrE* was obtained from *Thermosipho africanus* OB7 (DSMZ 5309) (137). A nucleotide blast of the *T. africanus pyrE* against *Tma pyrE* showed no significant matches with only a maximum length of contiguous homology of 8nts and was therefore preferable to *pyrE* genes from more closely related *Thermotoga* species because of its greater degree of divergence. A synthetic allele of the *T. africanus pyrE* gene was designed by codon optimization to match the codon preference of *T. maritima*. The second component for the divergent genetic marker was a strong promoter to drive gene expression. The *groESL*p promoter was selected for this purpose because it was shown previously to be constitutively expressed at 80 ºC (16). The *groESp* promoter was 196nt in length and encoded at chromosomal positions 532,037 - 352,233. It was positioned so that the start codon of the codon optimized divergent *pyrE* allele was 10nt from the SD sequence in the *groES* promoter region. The resulting divergent genetic marker was called *groESp::pyrE*$_{\text{Taf}}$.

4.5 Targeted disruption of *T. maritima* chromosomal genes. Inactivation of *araA* (TM0276) was then pursued using *groESp::pyrE*$_{\text{Taf}}$. Natural transformation of the *pyrE*-129 mutant used the suicide plasmid, pBL1322, containing 5’ and 3’ regions of *araA* respectively flanking the genetic marker and positioned in a divergent direction. Uracil prototrophs were recovered by liquid enrichments followed by clonal isolation on selective medium plates. PCR analysis using genomic DNA from four isolates with primers 5’TM0275 and 3’TM0277 that were complementary to sequences external to the *araA* segments present in plasmid PB1322, produced amplicons for all isolates that were larger than the wild type *araA* allele and consistent with the insertion of the genetic

marker (**Figure 2.4**). This prediction was confirmed by DNA sequence analysis that verified the presence of a 763 bp insertion of the *groESp*::*pyrE*$_{Taf}$ genetic marker at genome coordinates 290,534 within *araA*. Phenotypic analysis demonstrated the putative *araA* disruption mutants had lost the ability to catabolize L-arabinose but not maltose relative to the parental strain supplemented with uracil or the unsupplemented wild type strain (**Figure 2.4**). One of these isolates named PBL3022 was pursued further.

4.6 Contribution of arabinose catabolism to hydrogen formation. Pentose catabolism offers an important route for fermentative H_2 formation as it can be derived from lignocellulosic plant biomass. While such yields have been predicted using thermodynamic considerations (138) and experimentally by cultivation of wild type *Thermotoga* species on pentose sugars (5, 9, 21), it had not been shown whether the pentose phosphate pathway (PPP) was the primary route for this process. To measure directly the importance of the PPP for H_2 formation by fermentation of arabinose, it was necessary to use cell lines in which this metabolic pathway had been inactivated. H_2 yields were determined for the *araA* disruption mutant (PBL3022) and its Ara^+ parent cultivated in a complex medium containing added amounts of L-arabinose. Complex medium was used to support cell growth at a moderate level and to ensure that a lack of H_2 production did arise merely from metabolic inactivity. H_2 produced in the presence of added sugar was normalized to background levels produced in the absence of added sugar. H_2-mediated growth inhibition (6) was avoided by increasing the culture head space relative to the culture volume based on experimental reconstructions (21). At L-arabinose concentrations ranging up to 0.1% (w/v), the $AraA^+$ strain produced H_2 relative to added sugar at a molar ratio of 3.3, while no detectable H_2 was produced by the *araA*

mutant (**Figure 2,5**). The difference observed in H_2 production between the *araA* mutant and its $AraA^+$ parent across these concentrations of added sugar verify *in vivo* that isomerization of L-arabinose to L-ribulose catalyzed by L-arabinose isomerase is the primary route of arabinose catabolism leading to H_2 formation. The apparent ratio of H_2 formed to sugar added was consistent with values predicted by the combination of the oxidative and non-oxidative pentose phosphate pathways (21, 41).

5. Discussion

The establishment of a chromosome engineering method for *Thermotoga maritima* realizes the promise of extensive investments in structural biology and genomics of this organism (7, 18, 130-132, 139). *T. maritima* holds much promise for renewable hydrogen synthesis and due to its rapid growth at high temperature is an important option to advance the synthetic biology of hyperthermophiles. Here, a suite of essential methods including several predicated on prior studies (77, 127-129) were combined to create the first example in any *Thermotoga* species of allele replacement by targeted homologous recombination. In addition to the rescue of deleted and disrupted genes, and the insertion of synthetic alleles, the specific role of a metabolic pathway towards hydrogen synthesis was established. Pentoses including xylose and arabinose are major constituents of lignocellulosic material. Evaluation of arabinose catabolism using a genetic approach demonstrates the importance of the non-oxidative pentose phosphate pathway and its susceptibility for its manipulation to perturb H_2. Metabolism of pentoses by *T. maritima* and *T. neapolitana* has been proposed showing H_2 values close to the theoretical yield of 3.3 (21, 40, 41). Using genetic approach it could be shown that arabinose contributes to H_2 production. In addition, a correction for H_2 production arising from fermentation of

conventional complex medium additives (yeast extract and tryptone) was made and revealed that the true ratio of H_2 per mole of arabinose was 2.97.

The pentose phosphate pathway has been studied extensively for its role in energy metabolism and all the genes have been characterized in mesophilic bacteria, yeast, and mammalian cells (34, 35, 38, 140). This pathway also plays a role in the generation of nucleic acids, amino acids and reducing equivalents for the production of cell biomass (38). Because of the relevance of pentose sugars for biohydrogen synthesis using renewable feedstocks, this study using a genetic approach provides evidence for their specialized consumption through this pathway (21, 40, 41).

This study demonstrates that homologous recombination can be used to modify the *T. maritima* genome. It is thus relevant to reflect on the native hybrid recombination system in this organism. In particular *T. maritima* encodes an archaeal/eukaryal Mre11 nuclease instead of the bacterial *rec*BCD enzymes. Moreover, *Tma* does not contain the eukaryotic RAD50 however it contains the eukaryotic RadA (TM0199) and a potential RadB annotated as a hypothetical protein (TM0370)(7). It has been shown that MRE11 can be associated with RAD50 to actively bind DNA. This complex form a catalytic head that contains an ATP-stimulated nuclease and DNA binding activity that indicates its potential role in processing DNA double stranded breaks in *Tma* (7, 53, 101, 139). It was also found here that a minimum of 100nt was required for recombination and that a putative recombinogenic sequence (AGCGG) located at the 3' end of *pyrE* and upstream region of *pyrE* may play a role at recombination at that locus. Consistent with this Chi-like sequence from *B. subtilis*. In addition, recovery of the synthetic *pyrE* allele encoding synonymous codon changes, offers more flexibility to future efforts for engineering the *T.*

maritima genome. Finally, the recovery of homologous recombinants indicates the *T. maritima* hybrid recombination system is fully functional in catalyzing cross over events and offers a new experimental strategy for exploring the biochemistry of these unexpectedly paired recombination proteins. Additional essential features of the *T. maritima* genetic system include natural transformation a genetic marker and a constitutive promoter for genetic marker expression. To further simplify the recombination method, concerted efforts established the existence of natural transformation in *T. maritima* in contrast to a previous report (128). Since natural transformation was evident it is worth noting presence in *T. maritima* of genes likely to encode competence functions including homologs of *B. subtilis com*EA and *com*FC, *H. influenzae* DNA processing (*dpr*A) and type IV secretion system: (*pil*A). The *pyrE* gene was obtained from *T. africanus* because this species is also hyperthermophilic and thus ensures the encoded protein would also exhibit thermostability, and to reduce sequence identity and thereby avoid unwanted recombination between respective *pyrE* sequences (137). Finally, expression of the divergent *pyrE* gene relied on the native *groES*p promoter. The successful transcriptional fusion of this promoter enabling genetic selection by homologous recombination suggests that other divergent genes from *T. africanus* can be used to expand the genetic tools in *T. maritma*. Current applications of the *T. maritima* genetic system concern both metabolic and non-metabolic targets. They benefit from extensive comprehensive prior studies about the biology, molecular biology, biochemistry and metabolism of this bacterial extremophile.

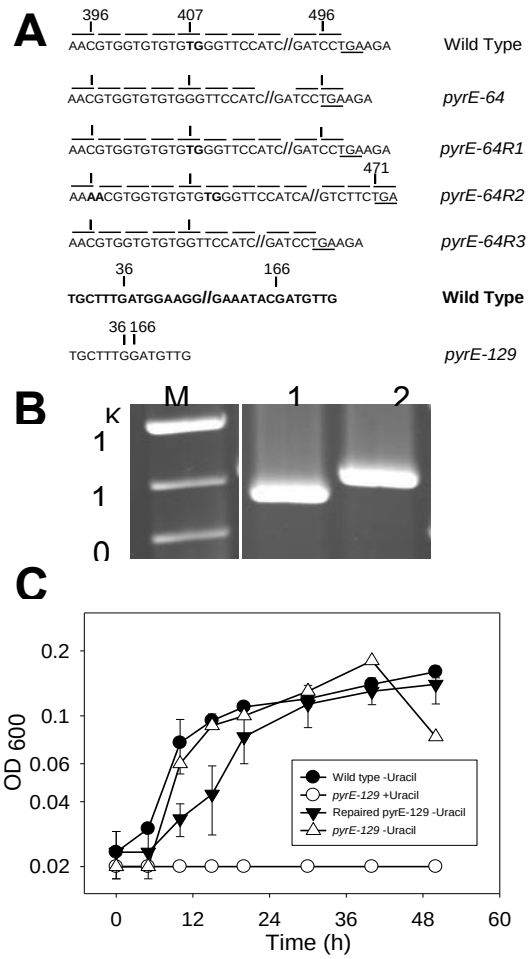

Figure 2-1. **Genotypic analysis, DNA sequence of, and growth curve of *pyrE-129* mutant**. (A) DNA sequence of *pyrE-64,* its revertants and *pyrE-129* mutants. The highlighted and boxed nucleotide sequence indicates site of the deletion and insertion events in mutant strains. The numbers indicate the location of the deletion and insertion within the *pyrE* gene in all five strains. (B) PCR amplification of the *pyrE* allele using genomic DNA from the *pyrE-129* mutant (lane 1) and the wild type (lane 2). (C) Growth of the *pyrE-129* mutant, the wild type, and the repaired *pyrE-129* mutant in a defined medium (DM) with and without uracil supplementation. The image of the gel was modified by cropping intervening lanes.

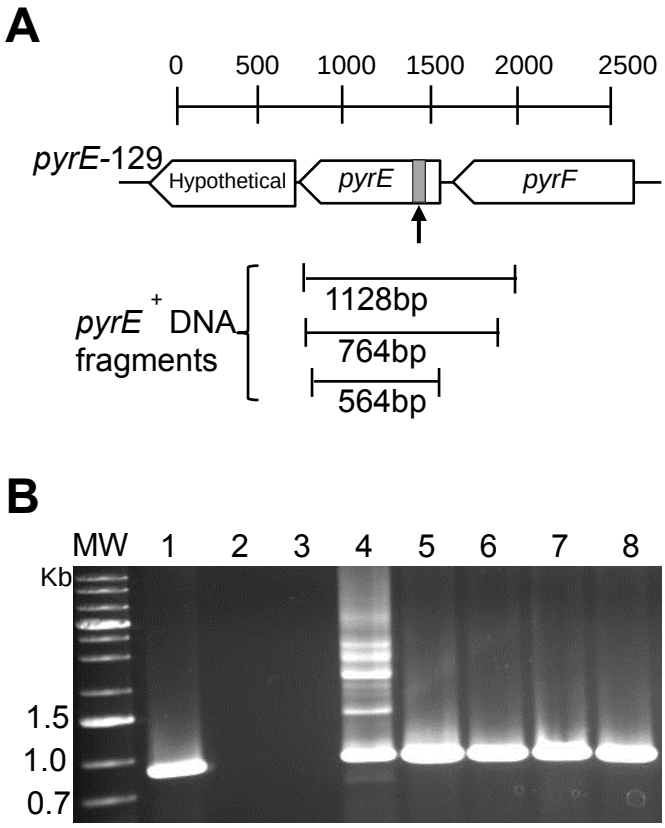

Figure 2-2. Schematic representation of the *pyr*E locus and wild type *pyrE* DNAs for repair of the *pyrE-129* mutant. (A) Genomic environment of the pyrE-*129* mutation. The gray bar indicates the location of the 129nt deletion in *pyrE*. The scale bar indicates 500 bp increments across the *pyrE* locus and the various length of wild type DNA fragments used for allele replacement. (B) PCR analysis of *pyr*E^{+} recombinants with a forward primer complementary to wild type sequence absent in *pyrE-129*. Lane 1: Wild type; Lane 2: *pyrE-129*; Lane 3: NO DNA; Lanes 4-8: *pyrE*$^{+}$ recombinants.

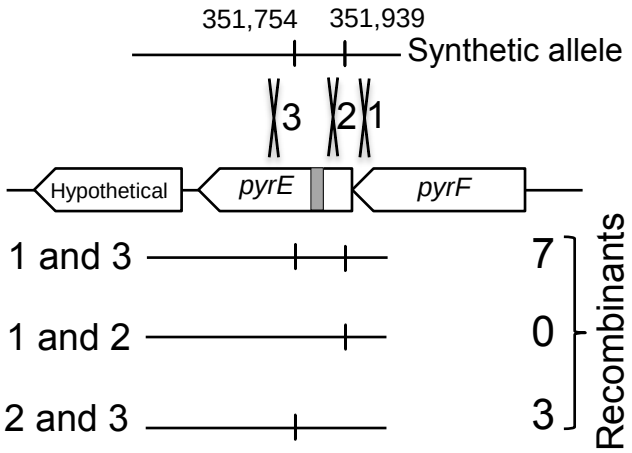

Figure 2-3: Recombination at the *pyrE* locus. Genomic region of the *pyrE-129* mutation is indicated by the tick marks with coordinates. The gray box indicates the location of the 129 nt deletion. The small black tick lines within the three lines indicate the location of the synonymous codon changes in the *pyrE* locus of the recombinants. The three Xs indicate the recombination events to give three recombination outcome when using the synthetic *pyrE* allele.

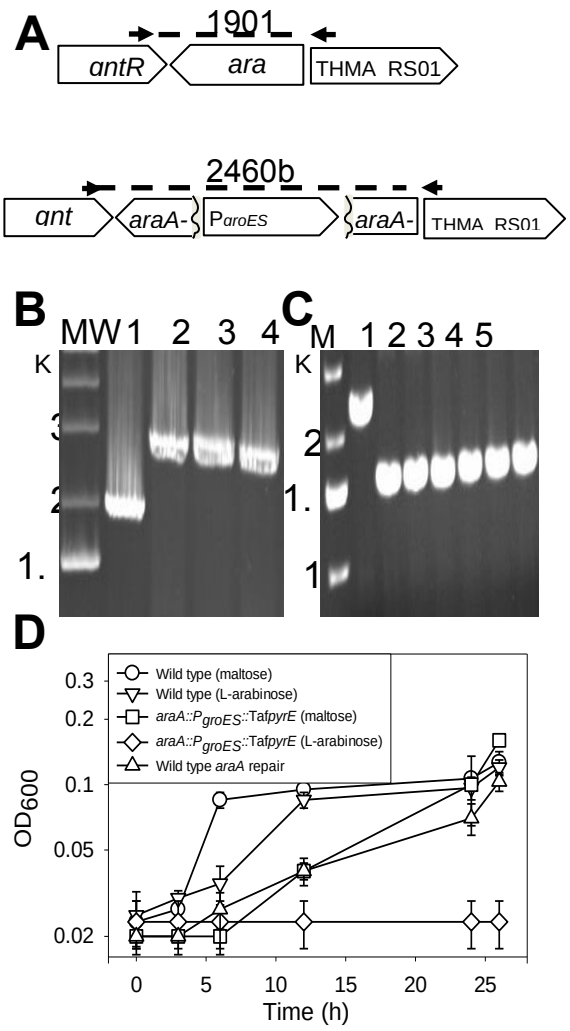

Figure 2.4: Disruption of *araA*. (A) Schematic representation of homologous recombination at the *araA* locus with the expected size for the *araA* mutant and the wild type using primers external to the *araA* locus. (B) PCR amplification of the disrupted *araA* allele. Lane MW, Molecular weight standards. Lane 1, wild type *araA* locus. Lanes 2-4, *araA* mutant locus. (C) PCR amplification of the repaired *araA* allele. Lane MW, Molecular weight standards. Lane 1, *araA* mutant. Lane 2, wild type. Lane 3-7, repaired mutant *araA* locus. (D) Growth curve of the *araA* mutant, wild type, and *araA* WT repair strain in defined medium with maltose or arabinose. Maltose was used as a positive control for growth**.**

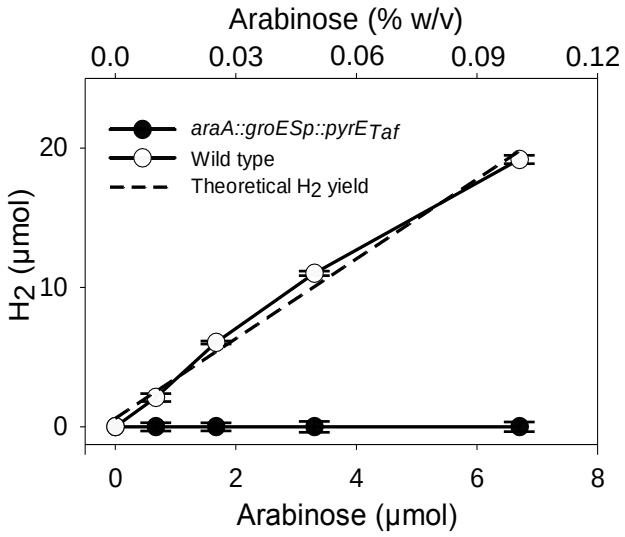

Figure 2.5: Analysis of H_2 production in the *araA* mutant. H_2 production by the *araA* mutant and parental strain cultivated with various amounts of arabinose. The straight line with the closed circles indicates H_2 production by the *araA* mutant. The dash line with the open circle indicates H_2 production by wild type. The H_2 values are shown as the mean of the results from three replicates, with the error bars representing the standard deviation. The dash line without symbols indicate the theoretical H_2 yield for growth on pentose sugar.

Table 2.1: *Thermatoga maritima* strains and plasmids

Strains	**Description**	**Source**
PBL3001	*Thermotoga maritima* MSB8	ATCC 45389 (26)
PBL3002	*pyrE-64*	PBL3001
PBL3003	*pyrE-64R1*	PBL3002
PBL3004	*pyrE-129*	PBL3001
PBL3005	*pyrE-64R2*	PBL3002
PBL3006	*pyrE-64R3*	PBL3002
PBL3020	*pyrE*$^+$ recombinant	PBL3004
PBL3021	*pyrE*$^+$ recombinant (synthetic *pyrE*)	PBL3004
PBL3022	*araA 3'*::P_{groES} *TafpyrE::araA 5'* mutant	PBL3004
PBL3028	*araA*$^+$ recombinant	PBL3022
Plasmids		
pBN1167	pUC19; WT *pyrE*$^+$ (564 bp)	This study
pBN1183	pUC19; WT *pyrE*$^+$ (1120 bp)	This study
pBN1290	pUC19; Synthetic *pyrE*$^+$ (977 bp)	This study
pBN1293	pUC19; WT *pyrE*$^+$ (764 bp)	This study
pBN1322	pUC19; *araA*3':: P_{groES} *TafpyrE* ::*araA*5'	This study
pBN1333	pUC57; WT *araA*$^+$ (1491 bp)	This study

Table 2.2: Primers

Primers	**Sequence**
pyrE 1F	GTGATAAAGGAAATCCTCGAGAAAA
pyrE 1R	TCATTTCAATCCCCTGCTTCCCGGT
pyrE 2F	GATAATCCTCGACCTGAAGTTCTGC
pyrE 2R	ACAGGTTCACTCGATTTTTCGACGA
pyrEF 3F	AGAGATGAAGGGAATAGCGAATTTT
pyrEF 3R	ACACCAGGGCACCTTTTATTATCAT
Synthetic *pyrE* 1F	GTGAAACTCACTTCCATGGAAGGAT
Synthetic *pyrE* 1R	TCACAGTCTCATCTCCTTTATC
pyrE 4F	TGGAAGGGCACTTCATTCTCTCTTC
pyrF 1F	ATGACACCTGTTCTCAGTCTGGA
pyrF 1R	TCACAGTCTCATCTCCTTTATC
groESL	CAGAAAGAGGGCGCTTCAAGCGCCT
Taf pyrE	TTATTTTTTAATGAATCTACTTCCT
THMA_RS01410	CGAGATCCTTTTGAAGATGATCGA
THMA_RS01420	CGAGATCCTTTTGAAGATGATCGA

CHAPTER 3

Evaluation of Iron Reduction by a Hydrogen Over-Producing strain of *Thermotoga maritima*

1. Abstract

Iron reducing microbes (FRM) play a vital role in iron cycling in both aquatic environments and sediments. This process has significant relevance because of its role in bioremediation, biomining, and other potential biotechnological application. While there is data showing iron reduction has been demonstrated by the hyperthermophilic bacteria *Thermotoga maritima* there is little known about this biochemical process. Using genetics along with an H_2 over-producing *T. maritima* stain (*Tma*200) we wanted to determine the mechanism for iron reduction and rate of iron reduction. Biotransformation of ferric (III) nitrilotriacetic acid (NTA) to ferrous(II) iron by was observed in a maltose dependent manner and verified using the ferrozine assay. *T. maritima* also was shown to form magnetic iron (magnetite) using an insoluble synthetic ferrihydroxide indicating *T. maritima* can donate electrons to a solid surface. *Tma200* exhibited the ability to reduce more soluble iron in batch cultures and under biofermentation conditions. Analysis of molecular hydrogen and organic acid showed a decrease in H_2 accumulation in the headspace and no detection of organic acid as compared to WT. This indicates that *Tma* excretes more reductant that is shifted toward the iron reduction instead of H_2 production and organic acid secretion in a sugar dependent manner.

2. Introduction

Dissmilatory iron reduction has been studied extensively in mesophilic bacteria namely *Geobacter* and *Shewanella* (102-104, 106). These mesophilic Fe(III)-reducing microbes play a significant roles in the bioremediation and bio-mining in mesophilic

environments. (141-146) Iron can also be found in hot environment suggesting iron can be used as an electron acceptor in these extreme conditions (116). The hyperthermophilic archaea that has been studied more recently is *Pyrobaculum* (116). Like the mesophilic Fe(III) reducing microbes *Pyrobaculum* species can used toxic metals and insoluble Fe as an electron sink (116, 147). Enzymes from *Pyrobaculum* that play a role in iron reduction have been identified and characterized (113, 147). *Pyrobaculum* species do not form pilins but some species have flagella and contain c-type cytochromes which have been reported to have key roles in iron reduction in *Geobacter* and *Shewanella* bacteria (148, 149). Dissimilatory iron reduction is considered to be common in hyperthermophilic archaea and soluble and insoluble Fe(III) reduction has been demonstrated in a hyperthermophilic bacteria *Thermotoga maritima,* yet little is known about how this reaction occurs (114).

Unlike the mesophilic Fe(III)-reducing bacteria and hyperthermophilic archaea iron reducers, *T. maritima* do not have genes that encoded for c-type cytochromes (7, 150). This suggests *T. maritima* may have a different mechanism for Fe(III) reduction that instead involves the type IV pilin gene, flagella, and Fe(III) reductase genes (7, 114, 150). With the capability to remove reductant by fermentation and its established genetic system makes it a preferable hyperthermophilic bacteria to address how iron reduction occurs in *T. maritima* (151-153). In this study we show an over producing hydrogen *T. maritima* strain that reduces soluble and insoluble iron faster as compared to the WT strain in batch and bio-fermentation conditions.

3. Materials and Methods:

3.1 Strains and cultivation. Unless otherwise indicated, *T. maritima* MSB8 (ATCC 45389, Genbank accession number NZ_CP011107) was cultivated at 80ºC under anaerobic conditions (131, 154). Strains of *T. maritima* are listed in **Table 3.1**. A

complex medium (CM) was prepared as described (154). For iron reduction experiments in bioreactors was done as previously described (not published) with modifications. Sodium sulfide was omittedfrom the Hungate tubes and bioreactors. Fe(III)NTA was added to the bioreactor as the electron acceptor. Fe(III)NTA and ferrihydrite was use for batch cultures.

3.2 *T. maritima* transformation. Transformations were conducted as previously described **(152).** For the preparation of cells for natural transformation, cells were processed as described (155) with modifications. A mixture of exponentially growing cells and plasmid DNA was added to 10 ml of CM and incubated at 80°C for 18 hours. Cells were collected by centrifugation, resuspended in 1ml of DM and adjusted to a cell density of 10^9 cells/ml by dilution. Cells (10^8/ml) were inoculated into selective medium for enrichment and then plated on defined medium plates to recover recombinants.

3.3 Plasmid and strain construction. A 5' flagellin fragment (582 bp sequence) was cloned at the pUC19 *Kpn*I restriction sites and a 3' *araA* fragment (582 bp sequence) was cloned at the pUC19 *Sph*I/HindIII restriction sites resulting in plasmid pBN1335 (**Table 3.1**). A 5' *pilA* fragment (538 bp sequence) was cloned at the pUC19 *EcoRIKpn*I restriction sites and a 3' *pilA* fragment (537 bp sequence) was cloned at the pUC19 HindIII restriction sites resulting in plasmid pBN1334 (**Table 3.1**).

3.4 Growth of *Tma* on Fe(III)NTA and Ferrihydrite. 1-10mM of Ferric (III) NTA or ferrihydrite from an anoxic bottled was added to growth medium as the electron acceptor. The controls were growth medium without the addition of maltose and no cells. The tubes were incubated at 80°C for until the brownish precipitate in tubes become clear or magnetite formation.

3.5 Ferrozine Assay for soluble and insoluble iron. The ferrozine assay was carried out on cultures supplemented with Fe(III)NTA that were incubated for 2-3 days at 80ºC. 5ml of 0.5N HCl was dispensed into scintillation vials. 250µl of samples was added to the 0.5N HCl. The samples were allowed to extract for 1hr when using Fe(III)NTA with gentle shaking on an Orbital Shaker in the dark. 1ml of Ferrozine was added to a cuvette then 25µl of the extracted samples were dispensed into the cuvette. The absorbance was immediately read at 562nm on a Cary 100 spectrophotometer. For ferrozine assay on cultures supplemented with ferrihydrite, the samples were allowed extract for 24hr in 3M HCl with gentle shaking on an Orbital Shaker in the dark when ferrihydrite was used. 1ml of Ferrozine was added to a cuvette then 25µl of the extracted samples were dispensed into the cuvette. The absorbance was immediately read at 562nm on a Cary 100 spectrophotometer

3.6 Hydrogen production. Molecular hydrogen (H_2) was analyzed using a Gow-Mac 400 series gas chromatogram equipped with a Molecular Sieve column (GOW-MAC, USA). Standard curves were prepared by injecting known amounts of H_2 with a bridge current of 90 mA. Temperatures used for the column, injector, and detector were 70°C, 90°C, and 90°C, respectively. Nitrogen (N_2) was used as the carrier gas at a flow rate of 30 ml/minute. H_2 in culture headspace was analyzed in triplicate and the error indicated. Growth of both strains in fermenters was conducted as previously described with modification to growth medium (not published). 10mM of Fe(III)NTA was added to growth medium and sodium sulfide was omitted from all bioreactor runs.

4. Results

4.1 Biotransformation of soluble and insoluble Fe(III). *T, maritima* grew in medium supplemented with soluble and insoluble iron. (Fig. 3.1) The Fe(III) was not visible in the culture tubes with sugar after 36hrs. When hydrogen was added as the electron donor without sugar as previously described (114) iron reduction was not observed (data not shown). Dissmilatory iron reduction was confirmed by detecting ferrous iron from cultures using the ferrozine assay. When sugar was removed from the tubes, the soluble Fe(III)NTA was visible in the tubes as compared to cultures supplemented with sugar and no cell controls (Fig. 3.1). This would suggest reductants generated from *T. maritma* during fermentation shifted towards iron reduction preferentially since hydrogen along with the drop in pH due to organic acid secretion inhibits the growth of *T. maritma*(11). Ferrihyrdrite was as reduced to magnetite. (Fig. 3.4) There was a significant chance in color (brown-reddish) of the Fe(III) oxide to black after 22 days as compared to the no cell control. The magnetic iron was validated by using a magnet (Fig. 3.4). TEM images show how the cells look after 22 days incubating with the Fe(III) oxide (Fig. 3.6). There seems to be appendages that extend from the *T.maritma* cells (Fig 3.6). This structure may play a role in attachment to the Fe(III) oxide and aid in electron transfer.

4.2 Effects of iron reduction on hydrogen production. As described above, hydrogen inhibits growth of *T. maritima* and we wanted to determine if hydrogen production was altered when cultures were supplemented with Fe(III)NTA. WT *Tma* was cultivated in complex medium supplemented with various amounts of Fe(III)NTA and a constant sugar concentration, hydrogen production decrease as the concentration of soluble iron increase. As predicted, the metabolism of *T. maritima* shifted when cultivated with iron as compared to controls without the addition of iron. Even though the addition of Fe(III) aided in the

reduction of hydrogen, its metabolism was not completely shifted toward iron reduction (Fig 3.3). Furthermore organic acid was not detected indicating that the metabolism may have shifted from organic acid production or formed a complex with the iron at high temperatures.

4.3 Targeted disruption of potential genes in electron transfer. Inactivation of *pilA* (THMA_1296, *fliC* (THMA_0777), the ferrodoxin-NADP reductase, and oxidoreductase (THMA_1681) was pursued. Since pilin and flagellin are associated with attachment and transfer of electrons to insoluble iron and metal surface (156-158), they were targeted first. Isolates were recovered by plating liquid enrichment on plates that did not require uracil. Five isolates were cultivated in defined medium without uracil supplementation for isolation of genomic DNA. PCR analysis show amplicons for all isolates were larger than the WT *pilA* and flagellin controls (Fig 3.5). This was consistent with the insertion of the selectable marker. Phenotypic analysis of *pilA* and flagellin mutant showed iron reduction using soluble and insoluble iron as compared to the WT controls. These findings indicatethat iron reduction may require other genes beside pilin and flagellin. The data for soluble iron reduction is consistent with previous data describe on *Geobacter* when *pilA* and flagellin is disrupted but *T. maritima pilA* and flagellin mutant were still able to reduce insoluble iron as compared to WT (156, 158). Other targets in *T. maritma* such as the the ferrodoxin-NADH reductase alpha subunit *sfrA* in *G. sulferreducans*was chosen because of its low homology with 45% identity. *Geobacter* also contains the *sfrB* gene which shared some similarity to the glutamate synthase in *T.maritima* (7, 159). The oxidoreductase was chosen due to its function in electron transfers and overlaps to the ferrodoxin-NADP reductase on the chromosome suggesting both genes maybe co-expressed. Efforts to

disrupt both alleles were unsuccessful indicating that these genes could be essential. Since *T. maritma* is a fermentative anaerobe that needs to remove reductants during fermentation, these genes may play roles in the metabolic pathways that are required for growth and other biological processes such as hydrogen production.

4.4 Effects on Fe(III) reduction by a hydrogen overproducing strain. In a previous report, a hydrogen over producing strain was isolated that passed the theoretical yield of 4 mol H_2/ mol of glucose (not published). Since this strain exhibited this phenotype, it was used to determine if ferrous accumulation would increase in tubes as compared to the WT control. Initial studies were carried out on batch cultures using different amounts of sugar while the Fe(III) was kept constant. When both strains were cultivated in growth medium supplemented with 0.5% maltose, there was no significant difference even though Tma200 reduced more Fe(III) as compared to the WT control (Figure 3.7). The reason for this result could be due to the excess of sugar added to the growth medium. To test this hypothesis, the sugar concentration was reduced to 0.1% maltose. The results showed that Tma200 reduced the soluble iron by 2-fold after 36 hours. Since WT *Tma* consumes sugar faster than Tma200, it will produce H_2 faster leading to Fe(III) reduction after 30hrs once the sugar is depleted. For the Tma200 strain, sugar uptake is slower allowing for more H_2 production at a slower rate leading to more ferrous accumulation in batch culture. This also suggests that more reductant is readily available for Fe(III) reduction by Tma200.

5. Discussion

This study represents an in depth evaluation of metal reduction by the fermentative hyperthermophilic bacterium *T. maritma*. The results show that *T. maritima* can reduce soluble and iron oxide when cultivated with sugar. Iron reduction was not achieved when sugar was omitted from growth medium supplemented with hydrogen. This indicates that

T. maritima does not conserve energy via dissimilatory iron reduction but as an alternative route to avoid hydrogen production which leads to growth inhibition. This is consistent with a reduction of hydrogen and when more soluble iron was added to growth medium. Previous reports on iron reduction in mesophilic and hyperthermophilic Fe(III) reducing microbes show that reduction is coupled to the use of electron donors such as formate or acetate while Pyrobaculum using hydrogen as the electron donor (102, 103, 110). These chemicals and gas was not added to *T. maritima* growth medium indicating that the generation of reductants via fermentation is the reason for the shifted toward iron reduction.

Attempts to determine genes required for iron reduction in *T. maritima* was unsuccessful. Even though the *pilA* and flagellin were disrupted, insoluble iron was still observed as compared to the WT. This findings indicates that the pilin and flagellin is not required for transfer of electrons to iron oxides as compared to *Geobacter*. Since *T. maritima* does not contain c-type cytochromes, the ferrodoxin-NADP reductase and oxidoreductase were targeted. We were unable to recover mutants indicating their importance in other biological processes that require these cofactors. There are other genes that could be targeted for disruption that may be responsible for iron reduction in *T. maritima*. There are reports of *P. carbinolicus* of the family *Geobacteraceae* ferments 2,3-butanediol and acetoin coupled to iron reduction as well as elemental sulfur required for reduction of Fe(III) (160). Under the growth condition described here sulfur was omitted from the medium showing *T. maritima* reduces both forms of iron using a different mechanism under fermentative growth conditions. Furthermore, *P. carbinolicus* also contain c-type cytochromes and geopilin of electroconductive nanowire that maybe involved in iron reduction (161, 162).

The analysis of reductant shifting towards iron reduction was tested using a hydrogen over producing strain described in(163). It was predicted that more ferrous iron would accumulation for *Tma200* as compared to the WT strains due to more hydrogen being generated due to an increase in the reductant pool. Iron reduction was determined using Balch tubes and under biofermentation conditions. Reduction of iron was improved when the *Tma*200 was used in batch cultures. The can be contributed to how each strain metabolized sugar in the presence of iron. The mutation in the *malK* gene allows for the slow uptake of sugar which allows for H_2 production to be produced slowly. This mechanism contributes to more iron being reduced over a longer period time as compared to the WT. With a functional *malK* in WT, the rate in which it takes up maltose is faster which leads to less hydrogen being produced required for iron reduction. Also the minimal amount of sugars was used by *Tma*200 to reduce more iron in batch cultures. From a biotechnological application stand point, this would potentially decrease cost for use on an electrode (115).

In this study, we showed *T. maritima* reduced both soluble and insoluble forms of iron by directed contact only when medium was supplemented with sugar. These results suggest that *T. maritima* does dissmilatory iron like the *Geobacter* and *Pyrobaculum* species but may use a different mechanism. This was demonstrated when the *pilA* and *fliC* gene was disrupted and the capacity to reduce insoluble iron was not lost as compared to WT and what has been described in *Geobacter*. There are other genes that could be targeted in *T. maritima* such as the NADH oxidase which transfer electron to oxygen. These genes could transfer electrons to both forms of iron under anaerobic conditions. *T. maritima* contains two NADH oxidases that have been isolated and

characterized and warrant further investigation as potential enzymes that may transfer electrons to soluble and insoluble iron (164). Finally, we show that a hydrogen over production strain of *T. maritima* could reduce more soluble iron using the minimal amount of sugar as compared to WT. The phenotype is of importance due to the amount of current *Tma*200 could generate for microbial fuel cells under conditions that do not require excess amount of a carbon source.

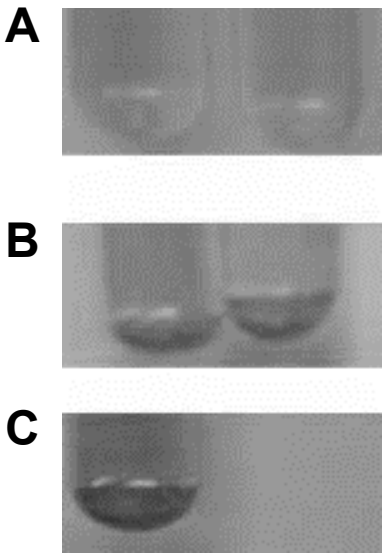

Figure 3-1. Biotransformation of Ferric NTA by *T. maritima*. The outcome of WT *Tma* into complex medium with and without maltose supplemented with 10mM Ferric(III) NTA. (A) *T.maritima*+maltose+Fe(III)NTA. (B) *T. maritma*+Fe(III)NTA. (C) No cells+maltose+Fe(III)TA. $1x10^{8}$ of cells was used as inoculum

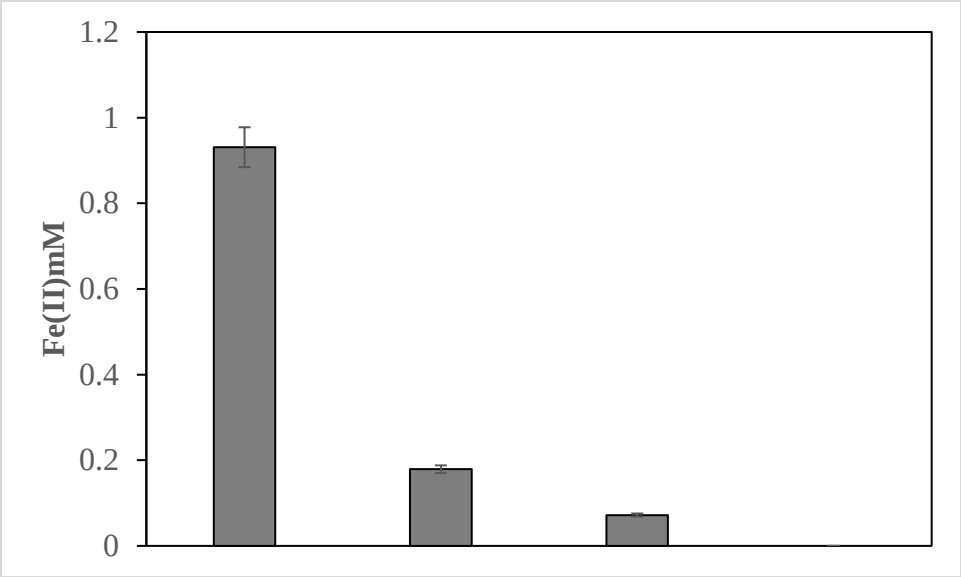

Figure 3-2. Conversion of Ferric(III) NTA to Ferrous(II) iron. Condition of tubes from left to right: cells+1mM Fe(III)NTA+maltose; 1mM Fe(III)NTA; 1mM Fe(III)+maltose with no cells; maltose only+cells. Ferrous accumulation was measured and confirmed by the ferrozine assay

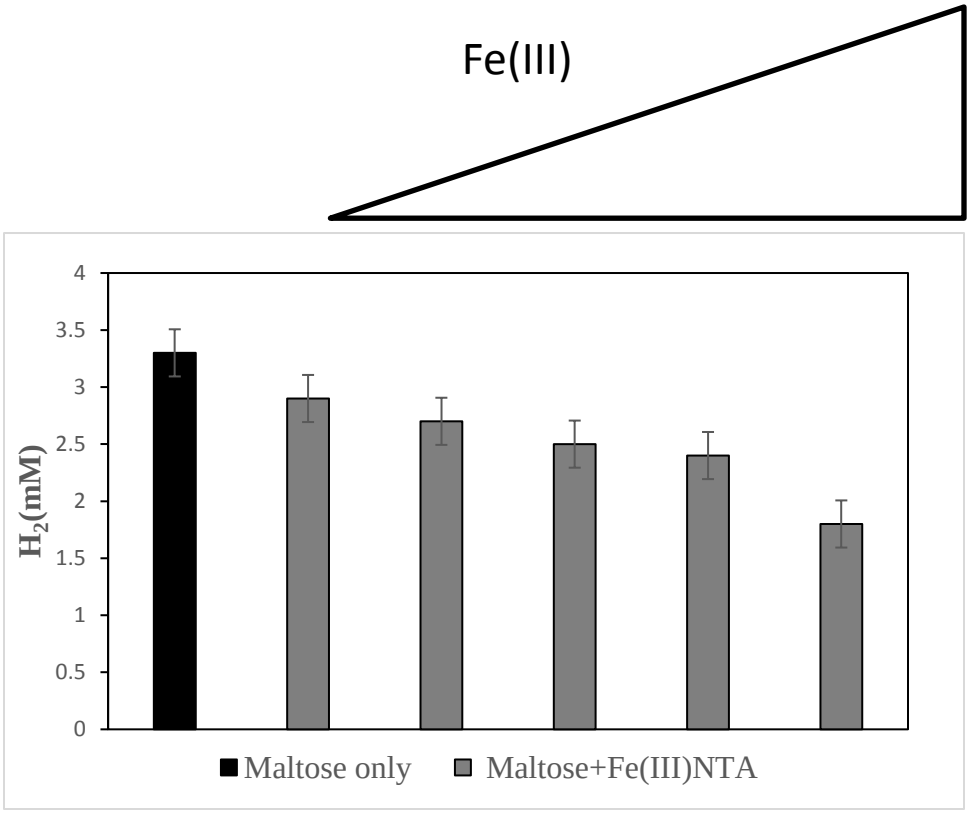

Figure 3-3. H_2 perturbation by *T. maritima* in soluble Ferrous(III) iron. H_2 synthesis by *Tma* cultivated in various concentrations of Ferric(III)NTA supplemented medium**.**

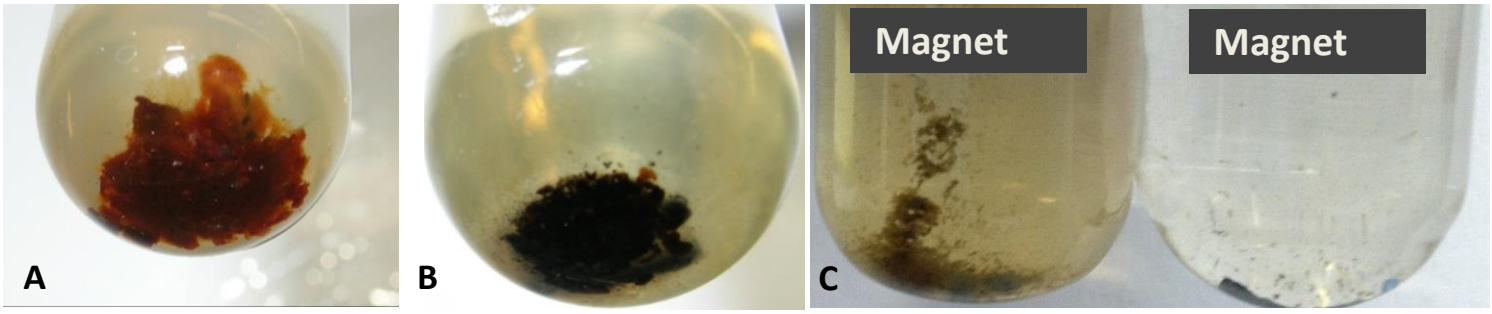

Figure 3-4. Reduction of insoluble ferrihydrite by *T.martima*. (A) *T. maritima* in medium supplemented with maltose and insoluble ferrihydrite prior to incubation. (B) Reduction of insoluble ferrihydrite by *Tma* after incubation is characterized by the color change from brown to black. (C) The magnetic property of magnetite is shown using a magnet.

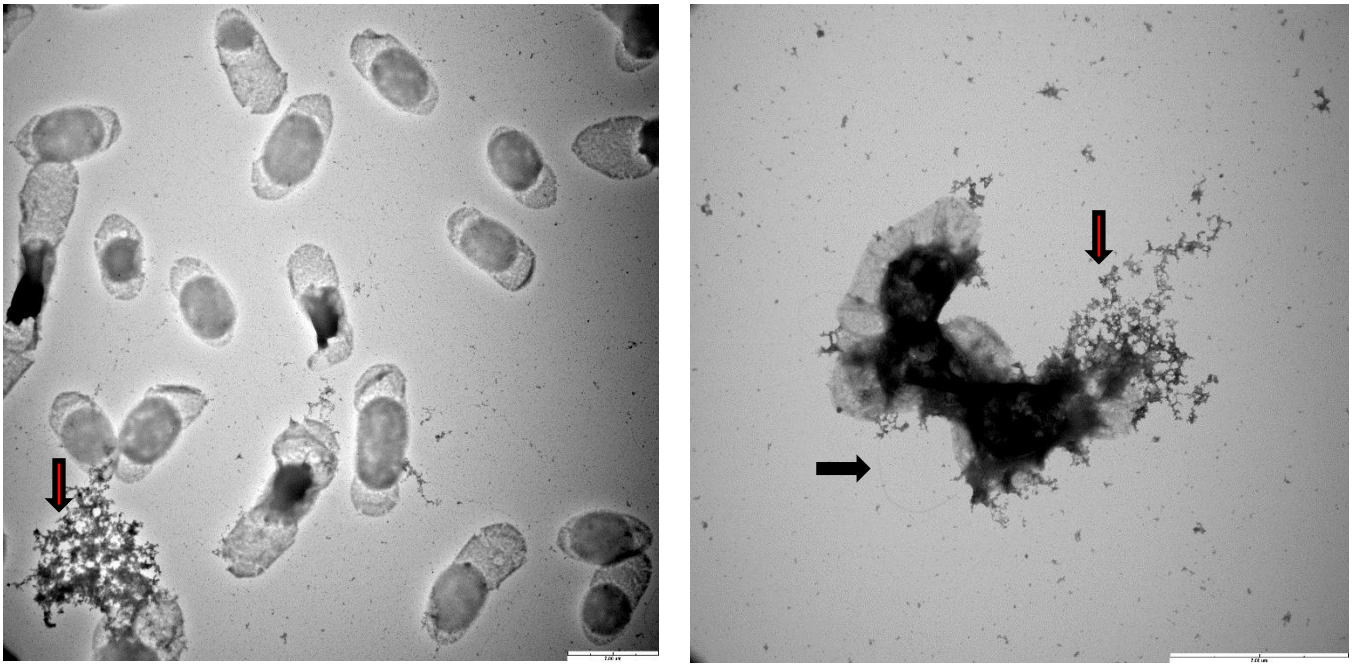

Figure 3.5. Transmission electron micrographs of *T. maritima* in insoluble Fe(III) supplemented medium. (A) *T. maritima* cells in growth medium containing insoluble Fe(III) before incubation. (B) *T. martima* cells after incubation at 80^{o}C for 22 days. Red arrow indicates the insoluble Fe(III). Black arrows indicated the position of the flagellum. Scale bars are 2.00mm

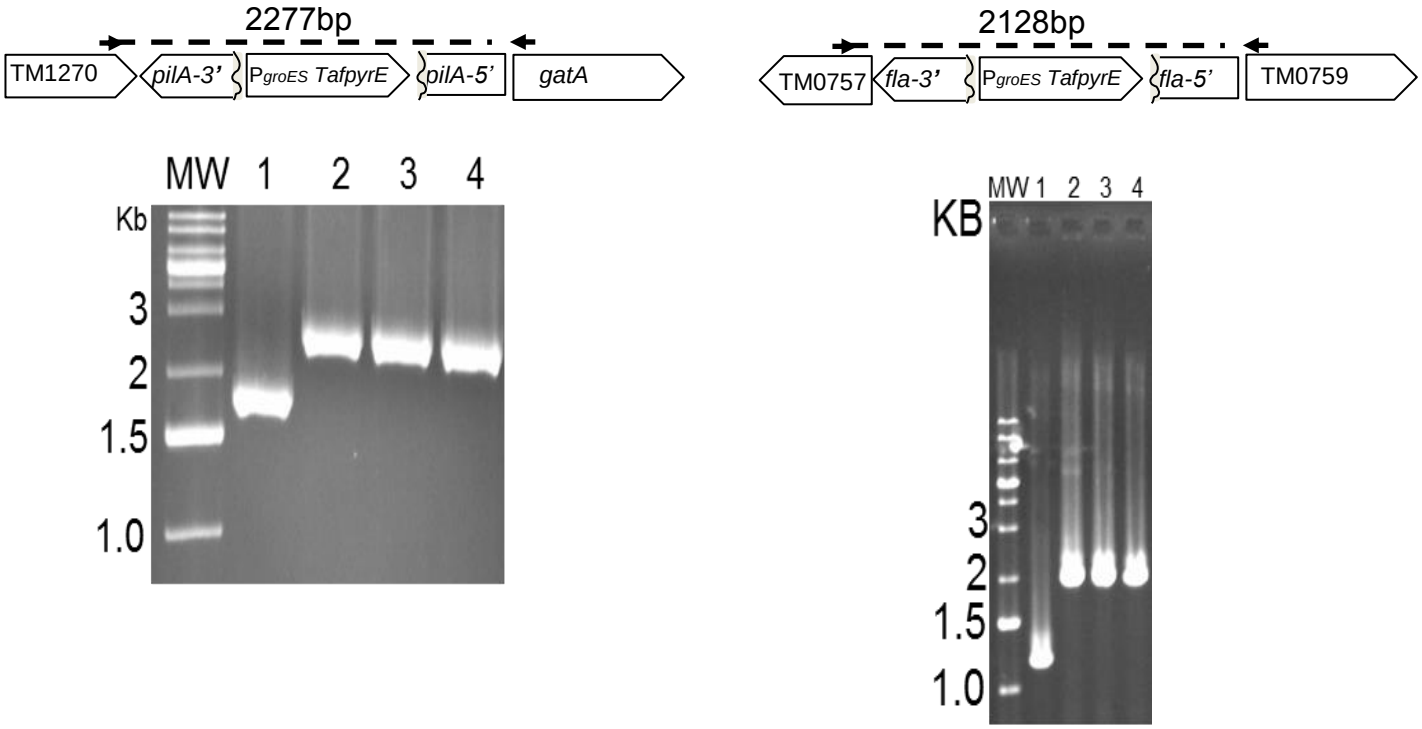

Figure 3.6: Disruption of *pilA* and flagellin. (A) Schematic representation of homologous recombination at the *pilA* and flagellin locus with the expected size for the *pilA* and flagellin mutants as compared to wild type using primers external to the *pilA* and flagellin locus. (B) PCR amplification of the disrupted *pilA* and flagellin alleles. Lane MW, Molecular weight standards. Lane 1, wild type *pilA* locus. Lanes 2-4, *pilA* mutant locus; (C) Lane MW, Molecular weight standards. Lane 1, wild type flagellin locus. Lanes 2-4, flagellin mutant locus.

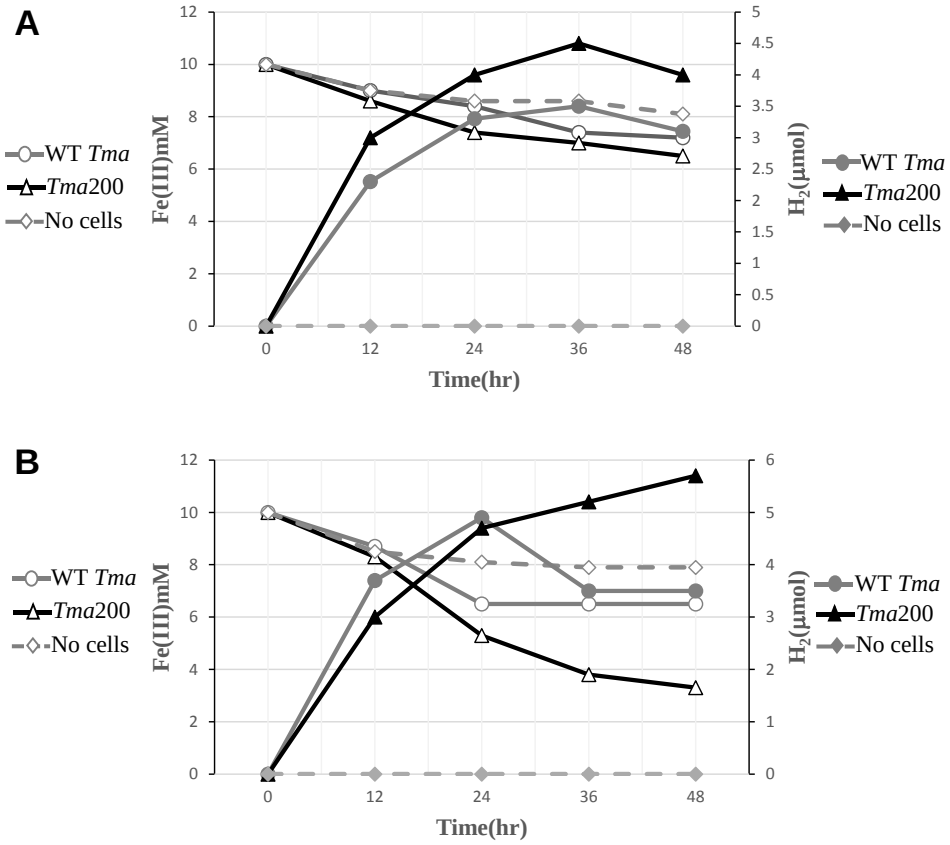

Figure 3.7: Reduction of soluble iron by *Tma200*and WT *Tma*. Reduction of 10mM Fe(III)NTA and H_2 production by ***Tma200*** and **WT *Tma*** in excess and limited maltose. (A) Ferrous accumulation and H_2 production by vs in complex medium supplemented with 0.5% maltose. (B) Ferrous accumulation and H_2 production by ***Tma200***vs **WT *Tma*** in complex medium supplemented with 0.1% maltose. The closed symbols indicates hydrogen production. The open symbols indicate the accumulation of ferrous iron.

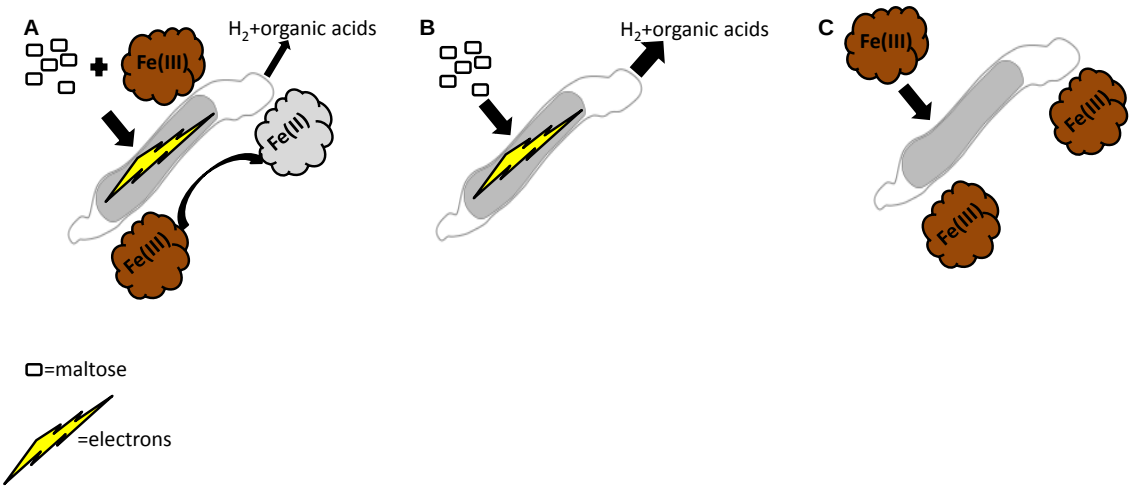

Figure 3.8: Schematic representation of iron reduction by *T.maritima*. (A) *T. maritima* in medium supplemented with maltose and Fe(III). (B) *T. maritima* in medium supplemented with maltose only. (C) *T. maritima* in medium supplemented with Fe(III) only. The square open box denotes maltose. The size of the arrows indicate the amount of hydrogen and organic acid production for each cultivation condition.

Table 3.1: *Thermatoga maritima* strains and plasmids

Strains	**Genotype**	**Source**
PBL3001	*Thermotoga maritima* MSB8	ATCC 45389
PBL3004	*pyrE-129*	PBL3001
PBL3029	*pilA 3'*::*groESp*::$pyrE_{Taf}$::*pilA 5'* mutant	PBL3004
PBL3030	*fla 3'*::*groESp*::$pyrE_{Taf}$::*fla 5'* mutant	PBL3004
Plasmids		
pBN1334	pUC19; *pilA*3'::*groESp*::$pyrE_{Taf}$ ::*pilA* 5'	This study
pBN1335	pUC19; *fla* 3'::*groESp*::$pyrE_{Taf}$ ::*fla* 5'	This study
pBN1336	pUC19; *fnr* 5'::*groESp*::$pyrE_{Taf}$ ::*fnr* 3'	This study
pBN1337	pUC19; *oxi* 5'::*groESp*::$pyrE_{Taf}$ ::*oxi* 3'	This study

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Appendix A:

Natural transformation of *Thermotoga maritima*

SOPs	Location 1	Location 2(Hard copy)
Complex medium	**Lab Share on computer; Derrick folder; SOPs folder**	**DJW Protocol binder**
Defined medium		
N_2 tank operation		
Frozen permanent preparation		
Bioreactor cultivation		
***Tma* cultivation on plates**		

Preparation of and shelf life for all components needed for growth of *Thermotoga maritima* in complex medium and defined medium are in the SOPs

Genbank accession number NZ_CP011107**:**
https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP011107.1

Protocol: Natural transformation of *Tma* uracil auxotroph

Comments: This protocol describes how to introduce DNA into the uracil auxotrophic mutant of *T. maritima* using natural transformation and how to select chromosomal recombinants resulting from repair of the *pyr*E mutation. Growth curves must be conducted to ensure use of cells at the appropriate growth state. Cells harvested at 0.4, 0.5, and 0.6 OD_{600}/mL can be used for natural transformation. All growth curves are to be started early in the morning.

MATERIALS REQUIRED
Micropipettors
*pyr*E mutant strain (PBL3004)
1.5mL microcentrifuge tubes
10mL complex medium in a Hungate tube
50mL complex medium in a serum bottle
20 ½G needle and 1mL cc syringe

60°C incubator (brown incubator by Bio Rad Gene Pulser)
55°C waterbath (located on the center bench in E223 lab)
10-20µg of plasmid DNA (plasmid prep fresh the day of transformation)
15mL defined medium in a Hungate tube
Defined medium plates (See Defined medium plate SOP)
Cary50 Spectrophotometer
15mL and 50mL Falcon Tubes
Paint cans or Almore jars (need information on how to use, close, gas packs etc)

PROCEDURE:
1. For all experiments the *pyrE* mutant culture must be inoculated from a frozen permanent. General use of *Tma* frozen permanents: DW/RS *Thermotoga* frozen- Shelf 3 Rack 3D; Main Thermotogales frozen stocks: Shelf 2-Rack 2D

2. Combine a $20^{1/2}$ G and 1cc syringe and remove 300µl of Complex medium from a sealed complex medium Hungate tube. Place the complex medium into a 1.5ml Eppendorf tube. From the -80C freezer on the second shelve from the top and the fourth row remove the box labelled "*Tma* Frozens" to use the tube labelled Uracil auxotroph - 129nt deletion: PBL3004. [See Extremophile book if you need to start PBL3004 from the master frozen] Remove cells by taking an autoclave stick in the test tube holder located by the -80C and scrapping cells from the tube. Add the cells to the 300µl and using the $20^{1/2}$ G and 1cc syringe remove the 300ml with cell and inoculate into a fresh 10ml complete medium hungate tube. Incubate the Hungate at 80C by placing the tube in the metal test tube holder.

3. Before using the Cary50 wash the cuvette with water and blank the Cary with complex medium at OD_{600}. For removal of inoculum from a serum bottle, combine a $20^{1/2}$ G and 1cc syringe then remove 1.5ml of inoculum from a sealed serum bottle and add to sterile 1.5ml Eppendorf. Take a P1000 and combine with one blue tip to take the inoculum from the 1.5 Eppendorf and place into the plastic cuvette and click the read button on the Cary50. Record the OD_{600} reading into your notebook. For use of the spec20, use a sealed complex medium tube to blank before use. If needed, change the test tube holder for the spec20 with a green piece of tape that indicates the proper alignment when putting it in. Move the knob on the top of the spec20 until you have it on 600. The knob on the front of the spec20 is used to zero the spec20. Once you have zero it out, take a kimwipe to clean the tube you will read then place it into the test tube holder. Cover the tube with the green kimwipe box near the spec20 and record the reading into your notebook

4. Sub-culture cells mid-log phase cells (0.4-0.5ODs) and take reading in the spec20. Transfer 100 µL into a Hungate tube containing 10ml complex medium. Incubate at 80°C in incubator located in the back room in E223 and place the Hungate tube in the metal test tube holder.

5. Take the OD_{600} reading of the Hungate tube on the Spec20. Subculture the cells when they have reached mid-log phase (0.4-0.5 OD_{600}) into a serum bottle containing 50ml complex medium at an initial OD_{600} of 0.02.

6. Measure the initial absorbance of the inoculated serum bottle at OD_{600} using the Cary 50. Use 1.5 mL of culture. Use fresh complex medium (aliquot the complex medium into a clean 10ml round bottom tube) and use as a blank. Incubate the culture at 80°C. Monitor growth by taking readings every 3 hr on the Cary 50 using complex medium as a blank.

7. When the culture in the serum bottle reaches OD_{600} of 0.4 or 0.5 OD_{600}, harvest the cells by centrifugation.

8. Transfer the remaining culture from the serum bottle to 50ml Falcon tube this will be approximately 45mL and centrifuge Sorvall RC5 centrifuge using the F21-8x50y rotor for 10 min at 6000 rpm at room temperature.

9. Decant the supernatant and re-suspended the cells in 1mL 20mM sucrose. This should result in a suspension with a density of 22.5 OD_{600} units/mL. Example: 0.5 OD_{600}/mL x 45mL=22.5 OD600 units in 1 mL.

10. Incubate the cells at 60°C for 20 minutes in the incubator located underneath the bench top with BioRad gene pulser on it. Before incubating the cells, transfer the suspended cells into a 1.5 microcentrifuge tube.

11. From the incubated cell suspension transfer 0.5ODs of cells (using the above example this would be 22 µL) into a 1.5mL microcentrifuge tube. Use separate microcentrifuge tubes of cells for experimental samples and negative control samples.

12. Add 10-20µg of the appropriate plasmid DNA encoding the genetic marker (*pyrE*) to the cells in the tube. Add nothing to the negative control sample of cells. Verify the concentration of the plasmid DNA stock sample using the Nanodrop and concentrations of 100ng/µL or less.
7/15/16(example)

Calculations:

Concentration	µl **needed to make 1-1.5µgs**
76.3	**?**

Examples:
76.3 ng/µl: **1 = 76.3**n**g**
76.3 ng/µl: **2 = 152.6**n**g**
76.3 ng/µl: **3 = 228.9**n**g**

Use 10-20µg to increase transformation efficiency for the positive control and experimental samples

13. Gently pipet the cells-DNA mixture once or twice to mix. Incubate the experimental and control cell samples for 30 min at 55°C in a water bath.

14. After the incubation period use a 20 ½ gauge needle attached to a 1cc syringe and remove 300 µL of complex medium from a Hungate tube and add it to the experimental and controls samples and transfer the samples into a Hungate tube containing 9.7mL complex medium. Incubate the tubes at 80°C for 18-24 hours.

15. After incubation check the cell density using the Spec 20 relative to uninoculated complex medium Hungate tube. The cultures should be at an OD_{600}: 0.5. Harvest the cells from the Hungate tubes (place the tube in a rubber adaptor) by centrifugation for 10 min at 6000 rpm at room temperature. Meanwhile, prepare tubes(1 control, 3 for experiments) for 1OD cells.

16. Decant the supernatant and re-suspend the cells in 1mL of defined medium. Do calculations for 1OD cell suspension before transferring into eppendorf tube. Then, transfer the cell suspension to a 1.5 mL microcentrifuge tube and wash them by centrifugation for 10 min at 8000 rpm, decanting the defined medium and re-suspending the cells in 1mL of fresh defined medium. This should result in a cell density of $1OD_{600}$ units/mL.

Calculations:

OD of tubes from Spec 20	**1OD of Cell Suspension needed**	**Defined Medium needed to make 1OD tubes**
0.39	**$1/3.9 = 0.2564 \approx 256\mu l$ of cell suspension needed**	**$1000\mu l - 256\mu l = 743\mu Ls$ of defined medium needed**

17. Using this suspension of cells, plate 0.2 mL volumes on defined medium plates. Inoculate 0.1mL volumes into Hungate tubes having 15 mL of defined medium with and without uracil at a final concentration of 40µg/ml.

18. Spot 10-serial dilutions of experimental, positive, and negative controls by serial diluting 10µl into 90µl of complex medium. This suspension (10^{-4} and 10^{-5}) is plated onto complex medium plates to determine the EOP and to provide a positive growth control.

19. Incubate all plates in a paint can with a EZ Gaspak then seal by placing the paint can lid onto the paint can and place a piece of thick wood on top of the lid. Using a hammer, hit the wood in a circular motion in order to ensure the lid is completely sealed) or use the Almore Anaerobic Jar using an EZ Gaspak and incubate at 80°C for 3 days.

20. Incubate the inoculated Hungate tubes with defined medium at 80°C and check them daily for the appearance of visible turbidity relative to the NO DNA control and by taking

OD_{600} readings on the Spec20 relative to NO DNA control tubes. Incubate the inoculated Hungate tubes with defined medium at 80°C for 2-3 days.

Safety: Discard the needles into the Red Sharp box located in the back room of E223 on the table between the 80C incubator and -80C freezer

After use of the N_2 tank, it is important to turn of the compressed gas after use because potential danger if knobs coming over if damaged and EHS violation if found on by EHS auditors.

Place all materials in which Na_2S into a labeled plastic bag. Place all used defined medium plates into a large 13 gallon clear garbage bag. When have full tag the 13 gallon bag for pickup by EHS.

Records and documentation: When preparing the *Tma* uracil auxotroph for natural transformation, make sure to label tubes and plates with the date, your initials, and which plates/or tubes contain uracil or no uracil. Date all solutions and record their use. This will help avoid use of expired reagents.

APPENDIX B

Preparation of complex medium liquid and plates for *Thermotoga maritima*

Protocol: Preparation of Complex Medium plates for *Thermotoga maritima.*

Objective: Complex medium liquid and plates (CM) are needed to for rapid growth and isolation auxotrophic mutants of *T. maritima*. This protocol details how to prepare complex medium liquid and complex medium plates for this purpose.

Safety: All laboratory personnel should be in compliance with UNL Employee Health and Safety regulations when working in the laboratory. Protective equipment, such as a lab coat and disposable gloves must be worn when working in the lab.
Records and documentation: When preparing complex medium liquid and plates, make sure to label the bag with the date, your initials, and whether the complex medium plates contain different sugars or antibiotics in them.
Date all solutions and record their use. This will help avoid use of out dated reagents.

Abbreviations and definitions: CM: Complex Medium; CMP: Complex Medium Plates.
MATERIALS REQUIRED

EQUIPMENT:
Sterile biosafety cabinet (tissue culture hood in Room E223A)
Micropipettors

SUPPLIES
Clean 1L bucket
Clean 2L Flask
Clean 1L Flask
Clean 250ml beaker
Clean 1L Graduate Cylinder
1 0.45µM sterile Filter
6 50ml Sterile Falcon Tubes
1 15ml Sterile Falcon Tube
2 sleeves of Biotang Plates (Sarstedt)
Clean stir bars

REAGENTS

Chemical	Catalog#	Supplier
Bacto Tryptone	211699	BD Biosciences
Bacto Yeast	212150	BD Biosciences
NaCl	5642-212	Fisher
Na_2SO_4	S-421-500	Fisher

$MgCl_2$	7791-18-6	Fisher
$NiCl_2$	N-5756	Sigma
Na_2WO_4	S-0765	Sigma
KI	P-8256)	Sigma
KBr	P-9881	Sigma
Boric Acid	B6768	Sigma
Resazurin	R-2127	Sigma
Maltose	M-5885	Sigma
$NaHCO_3$	7412	Mallinckrodt
Na_2S	52006	Sigma
KH_2PO_4	P285-500	Fisher
Phytagel	P-1869	Sigma

For preparation of Defined Medium, gelrite, and stock solution refer to Appendix I, II, & III

PROCEDURE

Complex medium plates

1. For preparation of 1L defined medium plates, take 500ml of defined medium (in 1L flask) and add to 500ml of gelrite (in a 2L flask) after autoclaving.

2. Stir the mixture at 450rpm on a stir plate for 5 minutes.

3. From the 75°C incubator, add the maltose and Na_2S+KH_2PO_4 mixture solution to the complex medium medium+gelrite mixture. **(Look at Appendix I for volume of each solution)**

4. Let the mixture stir for 5 minutes before pouring plates in the biosafety cabinet in E223A. If you use resazurin in preparation of plates, while mixing the mixture will become clear. Once the plates are poured, the color will turn pink indicating the presence of oxygen.

5. Let the plates sit overnight and use for plating the next day or store as needed at 4°C.

Note: complex medium plates have a shelf life of 2 months at 4°C.

REFERENCES

Thermotoga maritima defined growth medium was modified from Rinker's defined medium as described below:

Wolin, E.A., and Wolin, M.J., and Wolfe, R.S. Formation of Methane by Bacterial Extracts. 1963 The Journal of Biological Chemistry Volume 238, No 8.

Rinker, KD and Kelly, RM. Growth Physiology of the Hyperthermophilic Archaeon *Thermococcus litoralis*: Development of a Sulfur-Free Defined Medium, Characterization of an Exopolysaccharide, and Evidence of Biofilm Formation 1996 AEM Vol 62 No 12, pg 4478-4485

Rinker, KD and Kelly, RM. Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. 2000 Biotechnology and Bioengineering, pg 537-547

Appendix I: Preparation of Complex medium

Tma Media: Complex Medium Protocol

MW	Molarity	Ingredients	0.5Liter	1Liter
53.49g/mol	18.7mM	NH_4Cl	2.5g	5g
360.3g/mol	0.50%	Maltose	2.5g	5g
58.44g/mol	0.257M	NaCl	7.5g	15g
142.02g/mol	14.1mM	Na_2SO_4	1g	2g
203.3g/mol	9.84mM	$MgCl_2$ x $6H_2O$	1g	2g
84g/mol	2.98mM	$NaHCO_3$	0.125g	0.25g
119g/mol	0.168mM	KBr	10mg	20mg
61.83g/mol	0.323mM	H_3BO_3	10mg	20mg
166g/mol	0.120mM	KI	10mg	20mg
329.9g/mol	9uM	Na_2WO_4	1.5mg	3mg
237.69g/mol	8.4uM	$NiCl_2$ x $6H_2O$	1mg	2mg
251.2g/mol	3.9uM	Resazurin	0.5mg	1mg
	0.5%	Tryptone	2.5g	5g
	0.1%	Yeast	0.5g	1g

pH=7.0,autoclave

1. For 1L of complex medium plates, add 6g of Phytagel (Sigma) to 500ml of ddH_2O and heat on a hot plate with stirring until the phytagel goes into solution. Seal the top of the flask with aluminum foil while the phytagel is boiling.
2. Maltose, NH_4Cl, Na_2S, KH_2PO_4, 20 Amino acid solution, and vitamins are added after autoclaving.

Note: The location of each chemical can be found hanging below the chemical shelves in room E223A.

3. Addition of preferred sugar **(volume of concentration depends on how much sugar is required: i.e. final concentration of 0.5% maltose in 1L is 50ml)**. Aliquot

50mls of 10% Maltose into sterile 50ml Falcon Tubes. Aliquot 10ml of Na_2S and 1ml of KH_2PO_4 into a sterile 15ml Falcon tube.

4. Incubate all the tubes at 75°C once the gelrite and complex medium has been placed in the autoclave.

Note: The mixture is incubated at this temperature so the phytagel does not solidify when added after autoclaving. If you add cold solutions to the complex medium and gelrite mixture it will solidify and you will have to begin the entire process over.

If you forget to put the ingredients into the 75°C incubator and the autoclave process is complete, you can place both flasks into the 80°C incubation along with the ingredients. Incubate the tubes for 20 minutes. This will give the liquid time to heat up before pouring into the complex medium and gelrite mixture.

Appendix II: Preparation of Stock Solutions

Na_2S and KH_2PO_4 Stock Solutions.

10% Stock solutions are prepared for Na_2S and KH_2PO_4

1. For the preparation of 10% Na_2S and KH_2PO_4, in separate 200ml beakers, add 10g of either chemical to 50ml of ddH_2O.
2. Stir the mixture using a stir bar at 450rpm on a stir plate until the chemicals goes into solution.
3. Bring both solution up to 100ml with ddH_2O.
4. Filter sterilize the Na_2S into two 100ml serum bottles (50ml in both bottles) and seal with a blue butyl rubber stopper and aluminum seal. Remove the headspace with N_2 from both serum bottles. The serum bottles can be stored at room temperature
5. Filter sterilize the KH_2PO_4 into two 50ml sterile Falcon tubes and store the Falcon tubes at room temperature.

Note: Na_2S shelf life is 2 months under aerobic condition. (A green precipitate will form indicating oxidation of the solution and must be remade). Na_2S is filter sterilized into a 100ml serum bottle. The serum bottle is then sealed and headspace replaced with N_2. The appropriate amount of Na_2S is removed by a 22G1 syringe. Use the Bunsen burner at the bench top to flame the top of the serum bottle before removing Na_2S.

Note: KH_2PO_4 shelf life is 3 months under aerobic condition. A white precipitate will form in the tube when the solution is no longer usable. KH_2PO_4 is filter sterilized into a 50ml Falcon tubes and stored at room temperature in the 50ml Falcon Tube. **Final concentration of KH_2PO_4 in 1L of complex medium and defined medium: 7.3μMol**

APPENDIX C

Preparation of defined medium plates for *Thermotoga maritima*

Protocol: Preparation of defined medium plates for *Thermotoga maritima.*

Objective: Defined medium plates (DM) are needed to phenotype auxotrophic mutants of *T. maritima* and for the selection of chromosomal recombinants that repair *pyr*E mutations. This protocol details how to prepare defined medium plates for this purpose.

Safety: All laboratory personnel should be in compliance with UNL Employee Health and Safety regulations when working in the laboratory. Protective equipment, such as a lab coat and disposable gloves must be worn when working in the lab.
Records and documentation: When preparing defined medium plates, make sure to label the bag with the date, your initials, and whether the defined medium plates contain uracil or no uracil in them.
Date all solutions and record their use. This will help avoid use of out dated reagents.

Abbreviations and definitions: DM: Defined Medium; DMP: Defined Medium Plates.
MATERIALS REQUIRED

EQUIPMENT:
Sterile biosafety cabinet (tissue culture hood in Room E223)
Micropipettors

SUPPLIES
Clean 1L bucket
Clean 2L Flask
Clean 1L Flask
Clean 250ml beaker
Clean 1L Graduated Cylinder
1 0.45µM sterile Filter
6 50ml Sterile Falcon Tubes
1 15ml Sterile Falcon Tube
2 sleeves of Biotang Plates (Biotang Incorporated)
Clean stir bars

REAGENTS

Chemical	**Catalog#**	**Supplier**
NaCl	5642-212	Fisher
Na_2SO_4	S-421-500	Fisher
$MgCl_2$	7791-18-6	Fisher
$NiCl_2$	N-5756	Sigma
Na_2WO_4	S-0765	Sigma
KI	P-8256)	Sigma
KBr	P-9881	Sigma

Boric Acid	B6768	Sigma
Resazurin	R-2127	Sigma
Maltose	M-5885	Sigma
NH_4Cl	A-661	Fisher
$NaHCO_3$	7412	Mallinckrodt
Na_2S	52006	Sigma
KH_2PO_4	P285-500	Fisher
Nitriloacetic Acid	N9877	Sigma
$MnSO_4$ (monohydrate)	M-6528	Sigma
$FeSO_4$(monohydrate)	F7002	Sigma
$NiCl_2$ (hexahydrate)	N-5756	Sigma
$CoSO_4$ (heptahydrate)	C-6768	Sigma
$ZnSO_4$ (heptahydrate)	Z-4750	Sigma
$CuSO_4$ (pentahydrate)	C-7631	Sigma
Folic Acid	F-7876	Sigma
Pyridoxine-HCl	P-9755	Sigma
Thiamine-HCl	29F-0012	Sigma
Riboflavin	R-0508	Sigma
Nicotinic Acid	N-4126	Sigma
Biotin	B4501	Sigma
(DL)-Ca-panthothenate		
Vitamin B12	V-2876	Sigma
p-aminobenzoic acid	A-2905	Sigma
(DL)-6,8-thioctic acid		
Phytagel	P-1869	Sigma

For preparation of Defined Medium, gelrite, and stock solution refer to Appendix I, II, & III

PROCEDURE

1. For preparation of 1L defined medium plates, take 500ml of defined medium (in 1L flask) and add to 500ml of gelrite (in a 2L flask) after autoclaving.

2. Stir the mixture at 450rpm on a stir plate for 5 minutes.

3. From the 75°C incubator, add the maltose, $NHCl_4$, Na_2S+KH_2PO_4+Vitamin mixture, and 20 amino acid solution to the defined medium+gelrite mixture. **(Look at Appendix III for volume of each solution)**

4. Let the mixture stir for 5 minutes before pouring plates in the biosafety cabinet.

5. Let the plates sit overnight and use for plating the next day or store as needed at 4°C.

Note: Defined medium plates have a shelf life of two (2) weeks at 4°C, not longer.

Appendix I: Preparation of Defined medium and Gelrite

***Tma* Media: Defined Medium Protocol**

MW	Molarity	Ingredients	0.5Liter	1Liter
53.49g/mol	18.7mM	NH_4Cl	2.5g	5g
360.3g/mol	0.50%	Maltose	2.5g	5g
58.44g/mol	0.257M	NaCl	7.5g	15g
142.02g/mol	14.1mM	Na_2SO_4	1g	2g
203.3g/mol	9.84mM	$MgCl_2$ x $6H_2O$	1g	2g
84g/mol	2.98mM	$NaHCO_3$	0.125g	0.25g
119g/mol	0.168mM	KBr	10mg	20mg
61.83g/mol	0.323mM	H_3BO_3	10mg	20mg
166g/mol	0.120mM	KI	10mg	20mg
329.9g/mol	9uM	Na_2WO_4	1.5mg	3mg
237.69g/mol	8.4uM	$NiCl_2$ x $6H_2O$	1mg	2mg
251.2g/mol	3.9uM	Resazurin	0.5mg	1mg
pH=7.0,autoclave	final pH: 8.2			

1. Adjust the pH of the defined medium to pH 7.0 using 100% H_2SO_4, then autoclave. **(NOTE: BE CAREFUL WITH H_2SO_4. IT IS VERY CORROSIVE!!!!!!!!! USE LAB COAT AND GLOVES)**
2. For 1L of Defined medium plates, add 6g of Phytagel (Sigma) to 500ml of ddH_2O and heat on a hot plate with stirring until the phytagel goes into solution. Seal the top of the flask with aluminum foil while the phytagel is boiling.
3. Maltose, NH_4Cl, Na_2S, KH_2PO_4, 20 Amino acid solution, and vitamins are added after autoclaving.

Note: The location of each chemical can be found hanging below the chemical shelves in room E223.

Appendix II: Preparation of Stock Solutions

Na_2S and KH_2PO_4 Stock Solutions.

10% Stock solutions are prepared for Na_2S and KH_2PO_4

1. For the preparation of 10% Na_2S and KH_2PO_4, in separate 200ml beakers, add 10g of either chemical to 50ml of ddH_2O.
2. Stir the mixture using a stir bar at 450rpm on a stir plate until the chemicals goes into solution.
3. Bring both solution up to 100ml with ddH_2O.
4. Filter sterilize the Na_2S into two 100ml serum bottles (50ml in both bottles) and seal with a blue butyl rubber stopper and aluminum seal. Remove the headspace with N_2 from both serum bottles. The serum bottles can be stored at room temperature
5. Filter sterilize the KH_2PO_4 into two 50ml sterile Falcon tubes and store the Falcon tubes at room temperature.

Note: Na_2S shelf life is 2 months under aerobic condition. (A green precipitate will form indicating oxidation of the solution and must be remade). Na_2S is filter sterilized into a 100ml serum bottle. The serum bottle is then sealed and headspace replaced with N_2. The appropriate amount of Na_2S is removed by a 22G1 syringe. Use the Bunsen burner at the bench top to flame the top of the serum bottle before removing Na_2S.

Note: KH_2PO_4 shelf life is 3 months under aerobic condition. A white precipitate will form in the tube when the solution is no longer usable. KH_2PO_4 is filter sterilized into a 50ml Falcon tubes and stored at room temperature in the 50ml Falcon Tube. **Final concentration of KH_2PO_4 in 1L of complex medium and defined medium: 7.3 mmol**

2. Trace Element Stock Solution (filter sterilized: 1L of ddH_2O).

Chemical	**Amount**
Nitriloacetic Acid	1.5g
$MnSO_4$ (monohydrate)	0.5g
$FeSO_4$ (monohydrate)	1.4g
$NiCl_2$ (hexahydrate)	0.2g
$CoSO_4$ (heptahydrate)	0.36g
$ZnSO_4$ (heptahydrate)	0.1g
$CuSO_4$ (pentahydrate)	0.01g

Note: The stock solution of trace elements is good for 3 years. Store at 4C°.

3. Wolfe's Vitamins Stock Solution: (filter sterilized: 1L of ddH_20).

Chemical	**Amount**
Folic Acid	20mg
Pyridoxine-HCl	100mg
Thiamine-HCl	50mg
Riboflavin	40mg
Nicotinic Acid	50mg
Biotin	20mg
(DL)-Ca-panthothenate	50mg
Vitamin B12	1mg
p-aminobenzoic acid	50mg
(DL)-6,8-thioctic acid	50mg

Note: The stock solution of Wolfe's vitamins is good for 3 years. Store the serum bottle stock solution (O_2 removed from headspace with N_2) at 4°C wrapped in aluminum foil.

Appendix III: Preparation of 20 amino acid solution.

1. Prepare a 2mg/ml stock of 20 amino acid solution.

2. Final concentration of 20 amino acid solution should be 400µg. Add modest heat while the solution is stirring to reduce the time to solubilize the amino acids.

3. pH the 20 amino acid solution by added 800µl of 5N NaOH. The pH will be 7.

4. Filter sterilize the 20 amino acid solution by passing through a 0.45mM filter with a 60ml syringe into a sterile 50ml Falcon tube. You will need more than one 50ml Falcon tube.

5. Once all of the 20 amino acid solution is filter sterilized, place the 50 falcon tubes into a rack and incubate the tube at 75°C until needed.

Note: The mixture is incubated at this temperature so the phytagel does not solidify when added.

Note: 20 amino acid solution is made fresh (the same day the plates are made) each time before adding to defined medium and gelrite mix.

Note: The defined medium plates give the best efficiency of plating (EOP) when plates are prepared a day before plating of transformed cells. Plates are good for up to 3 months stored at 4°C

6. Addition of preferred sugar, NH_4Cl, Na_2S, KH_2PO_4, 20 AA, and vitamins. Aliquot 50mls of 10% Maltose and 10% NH_4Cl into sterile 50ml Falcon Tubes. Aliquot

10ml of Na_2S, 1ml of KH_2PO_4, and 1ml of vitamins stock solution into a sterile 15ml Falcon tube.

7. Incubate all the tubes at 75°C until needed.

Note: The mixture is incubated at this temperature so the phytagel does not solidify when added.

REFERENCES

Thermotoga maritima defined growth medium was modified from Rinker's defined medium as described below:

Wolin, E.A., and Wolin, M.J., and Wolfe, R.S. Formation of Methane by Bacterial Extracts. 1963 The Journal of Biological Chemistry Volume 238, No 8.

Rinker, KD and Kelly, RM. Growth Physiology of the Hyperthermophilic Archaeon *Thermococcus litoralis*: Development of a Sulfur-Free Defined Medium, Characterization of an Exopolysaccharide, and Evidence of Biofilm Formation 1996 AEM Vol 62 No 12, pg 4478-4485

Rinker, KD and Kelly, RM. Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. 2000 Biotechnology and Bioengineering, pg 537-547

Appendix D:

Analysis of Alpha-L-rhamnosidase activity in *Thermotoga petrophila* and adapted growth of *Thermotoga maritima* on rhamnose

Abstract

In an effort to introduce a gene in *T. maritima* that would confer a new trait, alpha-L-rhamnosidase activity was determined in *T.maritima* and *T. petrophila*. *T. maritima* does not contain an alpha-L-rhamnosidase gene so growth on dialyzed and undialyzed gelrite was conducted. *T. petrophila* was able grow on gelrite and dialyzed gelrite as compared to no growth of WT *Tma*. Initial growth experiments were conducted using the powder form of gelrite which allowed growth of both *Tpet* and *Tma*. This suggested that the powder form of gelrite may contain contaminants that allowed growth of both strains on gelrite. No growth was observed for *Tma* on dialyzed and undialyzed gelrite as compared to the positive growth growth control after a two day incubation period at 80°C. *Tpet* cells could be seen from experimental tubes (gelrite only) as compared to the positive growth control tubes indicating that there was preferential growth on gelrite. An increase in OD for *Tpet* cultivated in gelrite was also obseved using the spectrophotometer. Rhamnosidase activity was not observed in cell lysate for *Tpet* cultivated on 0.1% gelrite as compared to the positive (cells cultivated in rhamnose) and negative (cells cultivated in maltose). Integration of the native Tpet_1682 allele into the *pyrE*-129 mutant was unsuccessful. Since we did not use a codon optimized Tpet_1682 allele and the target for integration could have an impact on recovery of mutants that may contain the Tpet_1682 allele,

MATERIAL AND METHODS

Strains and cultivation. Unless otherwise indicated, *T. maritima* MSB8 and *T. petrophila* was cultivated at 80ºC under anaerobic conditions. Complex medium (CM) was prepared as previous described by (134). The carbon source was 0.5% maltose (Sigma) or rhamnose (Pfanstiehl).

Dialysis of Gelrite. To remove potential contaminants, 0.3% sterile gelrite solution was dialyzed in 1.5L of water twice using a Spectra/Por Dialysis Membrane (MWCO: 3500 daltons) then growth on the dialyzed and un-dialyzed gelrite was evaluated on both strains with positive growth controls in maltose or rhamnose (Sigma).

Alpha-L-rhamnosidase enzyme assay.

T. maritima and *T. petrophila* were cultivated in complex medium supplemented with 0.5% maltose or rhamnose until the cultures reached stationary phase (OD_{600} 0.6-0.9). Use 1ml of each culture and collect by centrifugation at 7000rpm for 15 minutes. Discard the supernatant and resuspend cell pellets in 500ml of 10 mM Tris-Cl pH: 7.0. Sonicate cells at 60W three times (30 second intervals) with 30 second incubations on ice. Place sonicated cells on ice and take a sample to view under the microscope to ensure the cells have been properly lysed. For the alpha-L-rhamnosidase enzyme assay, add 0.043g pNPR into 15ml of 100mM NaAc pH: 4.5 giving a final concentration of 10mM pNPR. After the addition of cell lysate to enzyme mixtures with controls, incubate the reactions for 30 minutes at 80°C. The reaction is stopped with the addition of 500ml of 1M Na_2CO_3 then take OD420 reading on a Cary50 spectrophotometer.

Calculating Activity. Add average OD_{420} of substrate only (tubes 2&3) + avg OD_{420} of cells+buffer (tubes 4&5). Subtract this value 1 from avg OD_{420} cells+substrate (6&7).

Multiply the value from 2 by 100 (μmoles of *p*NPR). Divide by 30 minutes. Resulting number = Activity (μmole *p*NPR/min)

Plasmid. The native Tpet_1682 open reading frame was cloned into the XbaI sites of pUC19. This insert was then flanked by insertion of *pyrE/F* and the hypothetical gene (TM0330) coding sequences. A 5' *pyrE/F* fragment (764bp sequence) was cloned at the pUC19 *Eco*R1 sites and a 3' TM0330 fragment (600bp sequence) was cloned at the pUC19 *Pst*I/*Sph*I resulting in plasmid pPB1322 Strain PBL3004 was then transformed to uracil prototrophy using DM lacking added uracil. Liquid enrichments were plated onto DM plates and incubated anaerobically at 80°C for three days. Genomic DNA was characterized for the *araA* allele by PCR using cultures prepared from colonies isolated from the selection plates cultivated in CM without selection for uracil prototrophy.

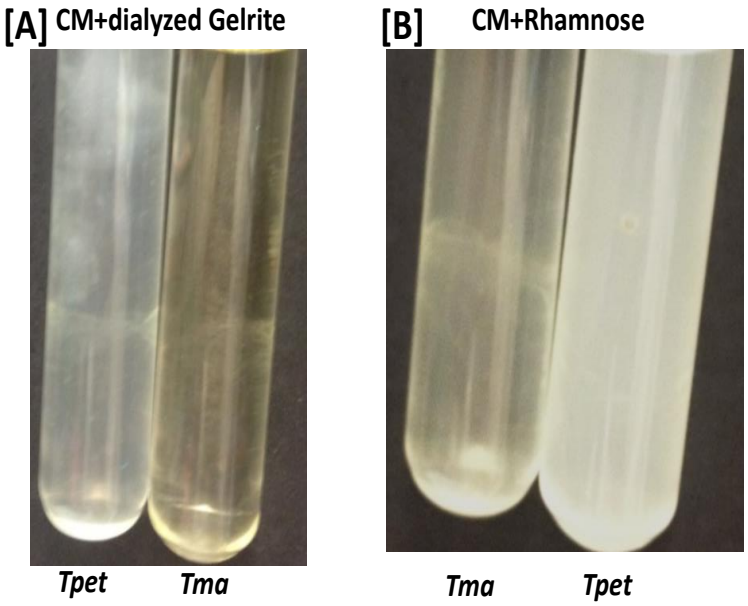


Figure 1: *T. martima* and *T. petrohila* grown in complex medium supplemented with gelrite or rhamnose. (A) Growth *of T. petrohila* on dialyzed gelrite with no growth of *T. maritima*. (B) Both strains growing in complex medium with rhamnose as the carbon source

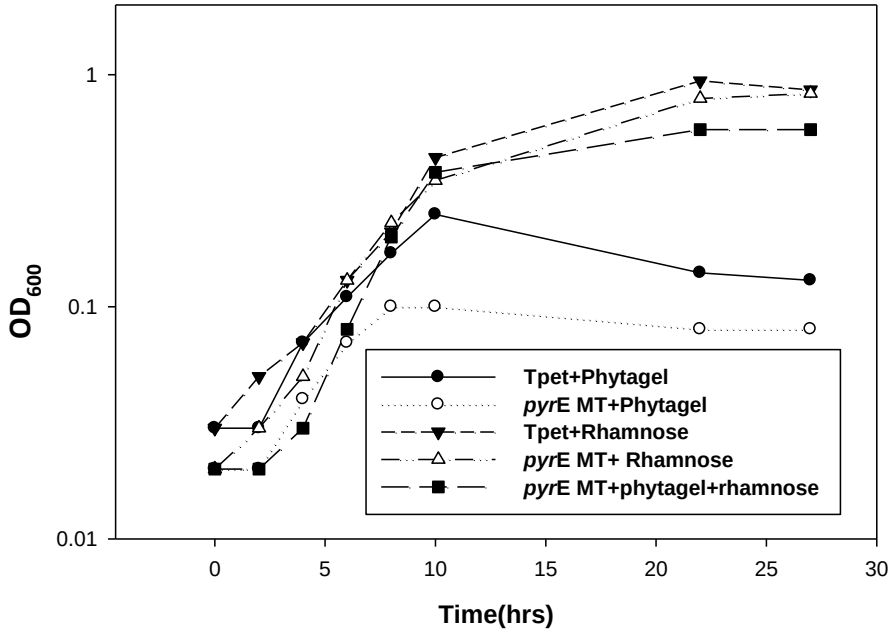


Figure 2: Growth curve of *T.petrophila* and *Tma pyrE* mutant on dialyzed phytagel

Growth of *Tpet* and *Tma* on Rhamnose and Gelrite				
Strains	Pre-growth	Sub-cultured into CM contain different carbon source	Growth VS No Growth	Rhamnosidase Activity
T. maritima	Maltose	Rhamnose+ Gelrite	No Growth	-
T. petrophila	Maltose	Rhamnose+ Gelrite	Growth	-
T. maritima	Maltose	Dialyzed Gelrite	No Growth	-
T.petrophila	Maltose	Dialyzed Gelrite	Growth	-
T. maritima	Maltose	Rhamnose	Growth	NO
T. petrophila	Maltose	Rhamnose	Growth	YES
T. maritima	Maltose	Maltose	Growth	NO
T. petrophila	Maltose	Maltose	Growth	NO
T. maritima	Rhamnose	Gelrite	No Growth	-
T. petrophila	Rhamnose	Gelrite	Growth	NO
T. maritima	Rhamnose	Dialyzed Gelrite	No Growth	-
T. petrophila	Rhamnose	Dialyzed Gelrite	Growth	NO
T. maritima	Rhamnose	Rhamnose+ Gelrite	Growth	-
T. petrophila	Rhamnose	Rhamnose+ Gelrite	Growth	-

Tpet_1682: Alpha-L-Rhamnosidase: Sequence Length: 2824bp: Hydrophobicity plot

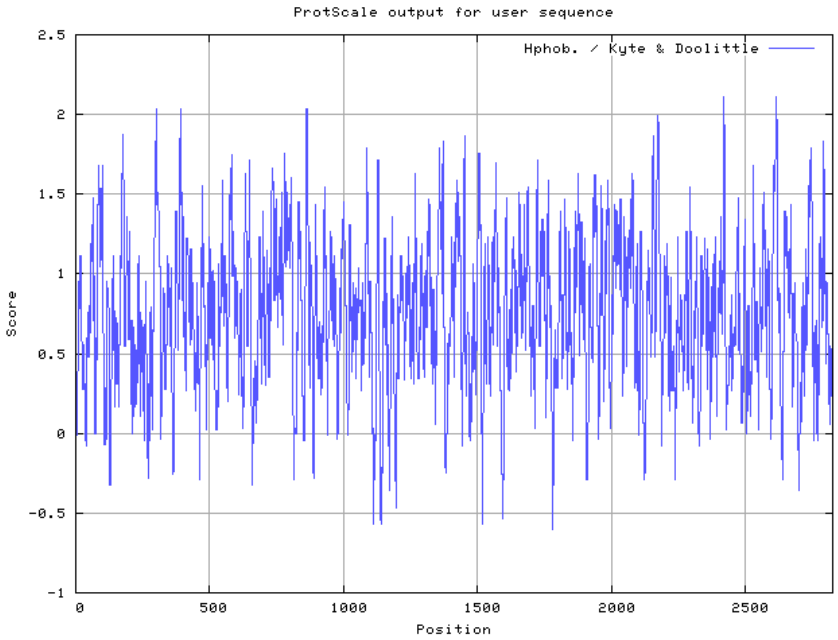
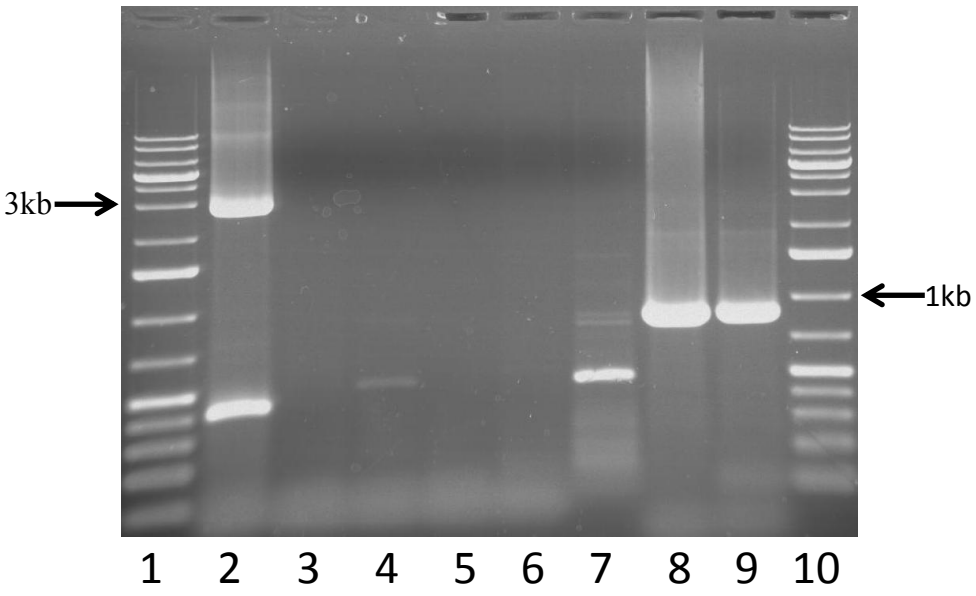


Figure 3: Hydrophobicity plot showing alpha-L-rhamnosidae potentially being secreted for degradation of alpha-L-rhamnose residues in alpha-L-rhamnosides



Lane 1: MM
Lane 2: Control (Tpet)(2.8kb)
Lane 3: pooled isolates
Lane 4: WT Tma
Lane 5: No DNA
Lane 6: Forward Primer Only
Lane 7: Reverse Primer Only
Lane 8: WT *Tma pyr*E allele (872bp)
Lane 9: pooled isolates *pyr*E allele
Lane 10: MM

Figure 4: The gel picture shows the amplification of *Tpet*_1682 from pooled isolates, *T. petrophila*, and WT *Tma* with *pyrE*. There was not amplification of the trans-gene from the pooled isolates as compared to the positive control

Future Direction:

These experiments were conducted in an effort to give *T. maritma* a new trait by expanding its utilization of carbon substrates. These are the potential experiments that need to be done since *T. maritima* has an established genetic system.

1) Construction of transcriptional fusion of *T. maritima* native *groES*p to the codon optimized Tpet_1682

2) Construction of *araA* disruption with *pyrE* in the backbone for p*yrE* recombinants then genotypic analysis of the *araA* locus for *groESp::Tpet*

3) Phenotypic analysis of Tpet_1682 recombinants on phytagel