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Engineering an anaerobic derivative of the obligately aerobic thermophile *Thermus thermophilus* HB28 for the purposes of studying thermophilic aerobic respiratory chain proteins

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Abstract

The thermophilic bacterium *Thermus thermophilus* (Tth) is an obligate aerobe with many important biotechnological applications and products. Thermostable proteins isolated from *Thermus* species have proven important in understanding adaptation to thermophilic environments. Thermophilic adaptations of respiratory electron transport chains (ETC) are not well characterized. Conventional homology-based, genetic, and biochemical techniques are limited in their ability to identify thermophilic Tth homologues of mesophilic respiratory ETC proteins due to the lack of a mutable facultative platform. We are attempting to generate this platform by increasing glycolytic flux through metabolic engineering techniques. Based on physiological studies, Tth HB27 demonstrates a low affinity for monosaccharide substrates, which limits glycolytic flux. We identify growth conditions suitable to increasing metabolic flux, as well as limitations to our current method for engineering a Tth mutable facultative platform. We also propose future direction in order to generate anaerobic derivatives of Tth HB27.

Introduction

Thermophiles: Introduction. Microorganisms are categorized into three divisions based on optimal growth temperature. An organism is classified as a psychrophile when it displays optimal growth at temperatures below 20ºC and as a thermophile when it displays optimal growth above 50ºC. Optimal growth between the limits of the psychrophilic and thermophilic designates a microorganism as a mesophile (9). Thermophiles are further subdivided into thermophiles and hyperthermophiles, with hyperthermophiles being capable of growth above 80°C (50). The majority of thermophilic bacteria grow optimally below the hyperthermophilic barrier (101).

Thermophilic microorganisms and their thermostable enzyme products are of great interest to industry and academic research due to their ability to maintain function and enzymatic activity at elevated temperatures. The development of recombinant DNA technology allowed a dramatic expansion in the study of thermostable enzymes, as well as spurring further interest in the isolation and characterization of novel microbes inhabiting thermal environments with the hopes of capitalizing on the commercial and industrial potential of thermostable enzymes (101). For example, the characterization and subsequent adaptation of *Thermus aquaticus* DNA polymerase for the amplification of DNA through the polymerase chain reaction has been one of the great commercial successes of thermostable enzymes (29, 73, 79).

Thermophiles: Habitats. Natural environments suitable for the growth of thermophiles, *i.e.* environments that keep stable temperatures in excess of 55°C, are limited primarily to hydrothermal vents and deep subsurface oil reservoirs (89, 85, 92). Thermophiles have also been isolated from various artificially heated habitats, including coal refuse heaps, geothermal power plants, compost piles, and hot water boilers (89, 91). Sporadically heated environments, such

as those generated by solar energy or the decomposition of organic occasionally permit the growth of thermophiles. However, due to the transient nature of these sporadically heated environments, growth conditions fluctuate between the optimal for thermophiles and mesophiles, leading to the evolution of microorganisms capable of growth under both thermophilic and mesophilic conditions (14).

Thermophiles: Metabolic and Molecular Adaptations. Environments with elevated temperatures pose a series of challenges to the growth of microorganisms. Most biomolecules are susceptible to heat. For example, as ambient temperatures are increased, phospholipid membranes become increasingly fluid and lose structural integrity, the DNA double helix melts into single strands, and proteins unfold as noncovalent interactions are disrupted. Owing to the particular problems of inhabiting an environment with elevated temperatures, and consequently low oxygen solubility, the majorities of thermophiles are obligate or facultative chemolithotrophic anaerobes and demonstrate various metabolic and molecular adaptations to elevated temperatures (79).

Most thermophiles display a respiratory metabolism, with aerobic or anaerobic respiration following inorganic reduction-oxidation reactions. Carbon dioxide serves as the primary carbon source while molecular hydrogen serves as the primary electron source. Some thermophiles also derive electrons from the oxidation of ferrous iron, sulfur, or sulfides. Oxygen serves as the primary terminal electron acceptor in aerobically respiring thermophiles, albeit primarily under microaerophilic conditions. Terminal electron acceptors for various anaerobically-respiring organisms primarily employ nitrate, carbon dioxide, ferric iron, sulfur, or sulfate as terminal electron acceptors. Both heterotrophic and autotrophic thermophilic microbes have been observed. Heterotrophic thermophiles can derive energy by either respiratory or

fermentative metabolic pathways (89, 91). Various novel metabolic pathways have also been observed in thermophiles and show promise as sources for novel enzymes and metabolic pathways that have not been observed in characterized mesophilic and psychrophillic organisms (46, 47, 111).

A range of novel molecular adaptations by thermophiles to cope with elevated temperatures have also been observed. These include adaptations at the genome, transcriptome, and proteome levels, as well as specific adaptations of structural macromolecules (38, 89). Specifically, growth at elevated temperatures requires adaptations such as increased RNA thermostability, increased stability of condon-anticodon interactions, and increased thermostability of enzymes, protein products, and cellular structures (79, 84). A detailed review of various genome and transcriptome-level adaptations to thermophily is given by Stetter, K.O., 2006 (90).

The adaptations of the proteome to growth at elevated temperatures are only beginning to be understood (38). While we can predict that proteins, due to their extreme thermolability (19, 42), would be subjected to intense selective pressures for structural stability at elevated temperatures suitable for the growth of thermophiles, we cannot yet predict with precision the result such selective pressures would have. While many studies have been carried out comparing thermophilic proteins to their mesophilic homologs (22, 38, 116), few truly universal principles governing the thermostable adaptation of proteins have yet to be determined (38, 77, 99, 116). However, it has been observed that when analysis is limited to genomes with similar nucleotide compositions, thermophilic adaptation of proteins consists of a significant reduction in the incorporation of the thermolabile amino acid residues histidine, threonine, and glutamine (27, 84), an increase in the number of negatively charged residues and positively charged residues in

approximately equivalent amounts (95), and a bias towards charged rather than polar residues (51, 93). On the proteome level, it has been broadly generalized that a greater fraction of proteins in thermophilic organisms have isoelectric points in the basic range compared to mesophiles (45), average lengths of thermophilic proteins are less than that of mesophilic proteins (51, 97, 115), and that there are a greater number of stabilizing salt-bridges and hydrogen bonds within thermophilic peptides (4, 17, 20, 52, 71). Protein chaperones in the form of heat shock proteins have also been characterized in thermophiles (82). In order to maintain protein function, thermophiles appear to devote more energy towards various methods of protein stabilization than do their mesophilic counterparts.

Elevated temperatures also pose a challenge for the maintenance of the integrity of phospholipid membranes. As temperature is increased, phospholipid membranes become increasingly fluid, eventually losing all semblance of a coherent structure. As a result, phospholipid membranes of thermophiles tend to contain more saturated fatty acids to help maintain the rigidity of the membrane and thus, appropriate function (79).

Thermophiles: Phylogenetics and Origin of Life. The development of techniques to directly analyze 16S and 18S rRNA sequences for the determination of phylogenetic relationships led to the development of a new hierarchical system for all living organisms: the domains Eucarya, Archaea, and Bacteria (68, 109). Thermophilic organisms are found in all three domains (Figure 1). In comparison with Bacteria and Eucarya, thermophily is quite common among the Archaea (38). Thermophily is relatively uncommon in Bacteria, and is extremely rare in Eucarya (38, 75). Phylogenetic studies have shown that thermophiles are among the most primitive organisms in existence, and that the universal common ancestor may be a thermophile (38, 54, 76, 90). Among the approximately 90 species of hyperthermophilic

Archaea and Bacteria that have been characterized to date, phylogenetic relations based on the analysis of gene involved in DNA replication, transcription, and translation parallel the conclusions reached by analysis of 16S rRNA sequences (90). However, analysis of genes involved in metabolism in characterized archaeal and bacterial thermophiles reveal frequent horizontal gene transfer events (21), accounting for the presence of archaeal gene sequences found in the genomes of bacterial thermophiles (54).

Thermophiles: Applications for Biotechnology. Since the discovery and characterization of *Thermus aquaticus*, the first thermophilic bacterium to be isolated from nature (10), the potential advantages of thermophiles and their gene products to both industry and academic research have been recognized. The previous identification of archaeal and bacterial thermophiles with characteristics desirable for both industrial and scientific purposes has led to an expansion in the study of and industrial application of thermophiles (2, 5, 14, 18, 43, 57, 78, 106, 110, 113, 114). Protein and nucleic acid thermostability and development of suitable industrial-grade thermostable enzymes are of particular interest (14, 89, 90), particularly since the commercial and scientific successes of thermostable DNA polymerases.

Thermostable enzymes are particularly advantageous over their mesophilic counterparts in biotechnological applications (29, 53, 102). Due to the challenges of maintaining function at elevated temperatures, thermostable enzymes are more resistant to thermal degradation, high salt concentrations, and acidic or basic conditions (56). There are several advantages to the use of thermostable enzymes in biocatalytic processes, as enzymatic biocatalytic reactions carried out at elevated temperatures have the advantages of increased enzymatic catalytic activity, lower viscosity, improved substrate solubility, and a reduced risk of microbial contamination (29, 43, 104).

Thermophiles: Suitability as Model Organisms. As previously discussed, there is great interest in the use of thermophilic prokaryotes in the laboratory. However, few characterized thermophilic prokaryotes are suitable as laboratory models, primarily due to the conditions under which these organisms grow. At the elevated temperatures required for their optimal growth, consideration must be given to issues with both laboratory equipment and the metabolic requirements of the selected model organism.

Common laboratory equipment, particularly those with electrical components, is sensitive to operation at elevated temperatures. The permanent magnets in orbital rotary incubator electric motors, particularly if not designed for high temperature applications, can suffer demagnetization and subsequent failure. Plasticwares commonly used in the culture of microorganisms, such as plastic Petri dishes, can warp or even melt. The dehydration and even melting of solid media can be problematic. While trivial in comparison to the above issues, the cost of both electricity and equipment degradation associated with maintaining high-temperature cultures must also be considered. Heat-resistant equipment, such as specially designed incubators, glass or Pyrex Petri dishes, and alternate solidifying reagents may be employed to counteract the above problems (14).

The metabolic requirements of thermophiles, particularly of anaerobic or chemolithotrophic organisms, can be problematic as well (103). In some cases, a thermophilic organism may also require other extreme conditions, such as high pH or high salt concentrations (60). The low growth rate of many of these organisms is extremely problematic (14).

While most thermophiles belong to the domain *Archaea*, only a few archaeal species have been subject to much genetic manipulation (2, 5, 18, 43, 57, 78, 110). Of these organisms, only *Thermococcus kodakarensis* was established as a decent laboratory model (78) due to the

development of efficient transformation and selection methods (14). However, *T. kodakarensis* required anaerobic growth conditions and elemental sulfur to maintain growth, which limits its potential useful applications. Among the few characterized *Bacteria* thermophiles, various strains of the thermophilic obligate anaerobe *Thermotoga maritima* were subjected to manipulation without much follow-up work (113, 114). With the discovery and characterization of *Thermus thermophilus*, it seems a suitable thermophilic laboratory model has been established (14).

The Genus *Thermus*. Organisms of the genus *Thermus*, first characterized in 1969 with the discovery of *Thermus aquaticus* (10), are Gram negative thermophilic bacterium. Most strains of *Thermus* are obligately aerobic, have high growth rates, and grow optimally between 62°C and 75°C (14). Some *Thermus* strains have been observed to be capable of anaerobic respiration when the growth media is supplemented with nitrogen oxides or metals (14). Morphologically, *Thermus* species are bacillar-shaped (Figure 2). Some strains demonstrate a tolerance for salt concentrations as high as 6% (66). Through the phylogenetic analysis of 16S rRNA and various protein sequences, bacteria of the genus *Deinococcus* have been determined to be closely related to bacterium of the genus *Thermus*, forming the phylum *Deinococcus-Thermus* (32, 33, 69, 108). Debate currently exists as to the precise phylogenetic location of this phylum (14).

***Thermus thermophilus*: Introduction and Metabolism.** *Thermus thermophilus* is a yellow-pigmented member of the *Thermus* genus first isolated from an undersea geothermal vent in 1974 (70). The rapid characterization of the *T. thermophilus* proteome (14) and the sequencing of the genome of the HB8 and HB27 strains of *T. thermophilus* (37, 58) has established *T. thermophilus* as an excellent model organism for structural biology studies (14).

As *T. thermophilus* is naturally competent (28, 49), as well as being easy to culture (70), an assortment of genetic tools, plasmids, and selection systems have been developed for it. As a result of this, along with all the advantages of working with thermophilic proteins, *T. thermophilus* is widely studied, particularly in the fields of structural biology, and holds particular promise in biotechnological applications (14).

Most *T. thermophilus* strains are obligate aerobes, although a few anaerobic strains have been tentatively identified (14). Due to the relative ease of studying *Thermus* proteins, *Thermus* respiratory chain proteins are one of the best characterized in all living organisms (63). Two terminal oxidases have been characterized: a caa_3-type oxidase expressed under conditions of high oxygen availability, and a ba_3-type oxidase expressed under conditions of oxygen limitation (23, 35, 36, 63).

A few strains of *T. thermophilus* have been tentatively observed to respire anaerobically by denitrification (15). This trait has been shown to be conferred by a DNA fragment, dubbed the "nitrate respiratory conjugative element", which encodes nitrate reductase and various proteins required for its activity (74).

T. thermophilus utilizes both protein and carbohydrate substrates for growth. Active transport systems, primarily ATP-binding cassettes, are used for the uptake of substrates (14, 37). *T. thermophilus* can synthesize all 20 common amino acids and various necessary vitamins and cofactors (14). *T. thermophilus* also synthesizes standard polyamines such as putrescine, spermidine, and spermine, as well as rare long and branched polyamines such as caldopentamine, caldohexamine, and tetrakis (3-aminopropyl) ammonium (14). Polyamines are implicated in the stabilization of nucleic acids at elevated temperatures (7, 40, 62, 96, 105).

Homologs of mesophilic enzymes involved in glycolysis have been identified in *T. thermophilus* (112), and the Embden-Meyerhof pathway appears to be functionally complete (81). Furthermore, enzymes of the pentose phosphate pathway and the tricarboxylic acid cycle have been identified (81). Amino acid uptake systems, operating primarily by sodium-symport, as well as various amino acid dehydrogenases, allow the metabolism of amino acids (81). *T. thermophilus* can also utilize monosaccharides as a carbon source, although pentoses are rarely metabolized (3, 41, 64). Fermentative growth has not been observed, despite the presence of an active gene encoding lactate dehydrogenase (81). An extensive review of *Thermus thermophilus* metabolism is provided in the work of Cava et al. (14) and Sharp et al. (81).

***Thermus thermophilus*: A Thermophilic Model Organism.** *Thermus thermophilus* has become established as the premier thermophilic model organism due to the development of various techniques to the genetic manipulation of *T. thermophilus.* As previously discussed, *T. thermophilus* possesses an extremely efficient natural competence system (6, 80), as well as being capable of intra-species conjugation (74), allowing the efficient and reliable transformation of *T. thermophilus* with recombinant DNA. The development of various plasmids suitable as cloning vectors, the identification of thermostable antibiotic markers, either naturally occurring or devised through direct evolutionary approaches such as kanamycin (55, 59), bleomycin, and hygromycin (11, 65), and various gene reporters and localization tools (14) has led to the extensive use of *T. thermophilus* in the characterization of thermostable proteins, as an recombinant DNA and protein expression host, and as a good candidate for various biotechnology applications.

T. thermophilus also displays various characteristics amenable to its use as a model organism. *T. thermophilus* does not have any special nutrient requirements (14). Being an

obligate aerobe, this allows the culture of *T. thermophilus* with standard equipment used in the culture of common laboratory microbes such as *Escherichia coli* with only elevated temperatures as a special requirement. The HB27 and HB8 strains of *T. thermophilus* display particularly rapid growth rates, reaching stationary phase within 7 hours or less (70). Such prodigious growth rates, as well as high optimal growth temperatures, help minimize contamination by allowing *T. thermophilus* to out-compete most thermophiles at elevated temperatures, while those same temperatures prove fatal to mesophilic organisms with faster growth rates.

As discussed earlier, *T. thermophilus* has also proven particularly important in the study of structural biology due to the relative ease in purifying its thermostable proteins (as compared to their mesophilic homologs), *T. thermophilus* has one of the best structurally characterized proteomes. Approximately 21% of its encoded proteins have determined structures, if not biochemical characterization (14). As a result, *T. thermophilus* and its proteins are extremely useful in the study of protein folding, allowing the testing of protein structure hypotheses.

***Thermus thermophilus*: Applications for Biotechnology.** The same characteristics that make *T. thermophilus* strains excellent model organisms are also valuable for biocatalytic processes. Improved thermostability over mesophilic homologs, as well as resistance to high salt concentrations, acidic or basic conditions, etc. allows their use in a larger variety of reaction conditions more amenable to the biocatalytic process than a mesophilic homolog would allow. Particularly because of the ease of the ease genetic manipulation of *T. thermophilus* and purification of protein from it, *T. thermophilus* has been extensively "mined" for thermostable enzymes, as well as being utilized as a cell factory and a thermostable protein expression host (14).

Energy Utilization and Respiratory Pathways. Prior to the massive increase in atmospheric oxygen levels during the Paleoproterozoic brought about by photosynthetic organisms, anaerobic organisms dominated the planet (13). Due to low atmospheric concentrations of oxygen, any use of oxygen as an electron acceptor would have been rare (31). However, the development of aerobic respiratory metabolic processes prior to the so-called oxygen catastrophe cannot be discounted (31). Regardless, the rapid increase in atmospheric oxygen levels, combined with the development of efficient aerobic respiratory pathways, ago led to extinction of most anaerobic organisms and the subsequent establishment of aerobic organisms as the dominant life form on the planet (26).

Cellular respiration is comprised of three primary metabolic processes: glycolysis, the citric acid cycle, and oxidative phosphorylation (12). Glycolysis is an anaerobic process present in all living organisms, and derives two molecules of pyruvate, two molecules of adenosine-5'-triphosphate (ATP), and two molecules of reduced nicotinamide adenine dinucleotide (NADH). In the absence of oxygen, fermentation occurs, where energy in the form of ATP is derived from the oxidation of pyruvate with various endogenous electron acceptors, resulting in incompletely oxidized waste products such as ethanol or lactic acid. Fermentation is less efficient than aerobic respiration (12).

Decarboxylation of pyruvate into acetyl-CoA and carbon dioxide allows the utilization of the citric acid cycle. The citric acid cycle, also known as the tricarboxylic acid cycle or the Krebs cycle, is a series of chain reactions that generates two molecules of ATP or guanosine-5'-triphosphate (GTP), six molecules of NADH, and two molecules of twice-reduced flavin adenine dinucleotide ($FADH_2$). NADH and $FADH_2$ act as electron carriers, shuttling electrons (and thus, energy) to the enzymes of the oxidative phosphorylation pathway (12).

The oxidative phosphorylation pathway is comprised of a series of successively more electronegative metalloprotein complexes embedded in a membrane. Electrons donated by NADH and $FADH_2$ move through the series of metalloprotein complexes, providing the energy required to establish a proton gradient. Eventually, these electrons are used to reduce a terminal electron acceptor. In the case of aerobic respiratory pathways, this terminal electron acceptor is oxygen, and the process is catalyzed by cytochrome c oxidase. In anaerobic respiratory processes, another exogenous electron acceptor is used, such as nitrogen oxides, sulfur compounds, or ferric iron (12, 39). An extensive review of respiratory protein complexes can be found in Hosler, J.P. et al, 2006 (39).

Cytochrome c oxidase. Cytochrome c oxidase (CcO) is a multimeric integral membrane protein with several metal prosthetic sites (88). Most CcOs belong to the heme-copper superfamily of oxygen reductases, which is characterized by a low-spin heme group in subunit I and high spin heme and copper ion binuclear oxygen-reducing center (24, 61). CcO functions as the terminal electron acceptor in the electron transport chain of the oxidative phosphorylation pathway, reducing oxygen and maintaining the proton gradient (12).

Reduced cytochrome c docks on the positively charged side of the membrane and transfers electrons to the internal binuclear heme-copper oxygen reduction site (heme a_3/Cu_B) via the bimetallic Cu_A site located near the membrane surface (Figure 3a). The precise mechanisms of electron transfer through CcO are treated in more detail by Belevich, I. et al, 2006 (8), while the structure of the Cu_A and Cu_B sites are treated in more detail by Sousa, F.L., et. al, 2008 (88) and Sujak, A., et al, 2007 (94), while assembly of the Cu_A site is covered extensively by Abriata, L.A., et. al, 2008 (1). Biogenesis of bacterial respiratory chains is described in detail by Thony-Meyer, L., 1997 (98).

T. thermophilus encodes two heme-copper oxygen reductases, the caa_3 CcO and the ba_3 CcO. The *T. thermophilus* ba_3 CcO has been extensively characterized in terms of its kinetics (30, 44, 48, 83), biochemistry (36, 67, 117), and three-dimensional structure (36, 87), while the aa_3 CcO has been the subject of less inquiry (72). Of particular interest is the examination of the heme-copper groups in the *T. thermophilus* ba_3 CcO in order to determine mechanisms, adaptations to thermophily, and method of assembly.

Models of heme-copper site maturation. The assembly of respiratory chain proteins, CcO in particular, is complex multistep processes involving many diverse factors operating in a coordinated fashion (25). While the structure of CcOs have been determined (36, 87, 100) and biochemistry of CcO extensively characterized (36, 25, 30, 44, 48, 67, 83, 117), many questions still remain (72). Of particular interest is the transport and insertion of copper into the CcO apoprotein. Due to the cytotoxic effects of unbound copper ion radicals, copper chaperones have been determined to bind free copper and transport copper to their target sites in the CcO complex (25). In eukaryotic models, the incorporation of copper to the Cu_A and Cu_B sites of CcO is mediated by the cuproenzymes COX17, SCO1, and COX11 (Figure 3b). In summary, free copper ions are first bound by COX17, then transferred to SCO1 for subsequent loading of the Cu_A site or transferred to COX11 for loading of the Cu_B site (25). While mesophilic eukaryotic and prokaryotic analogs of this copper loading pathway have been characterized, a thermophilic analog has yet to be identified.

Problems with various methods to identify copper chaperones. The function of CcO is entirely dependent on the presence of the heme-copper groups. Previous work has determined the presence of the Cu_A and Cu_B sites within *T. thermophilus* CcOs (1, 72, 88). Due to the cytotoxic properties of free copper ions, we postulate the existence of copper chaperones

analogous to that found in mesophilic organisms in *T. thermophilus*. However, common approaches to characterizing novel enzymes, such as homology-based searches, biochemical techniques, and genetic techniques all have inherent disadvantages or technical limitations when applied to *T. thermophilus* and its putative copper chaperones.

Homology-based searches rely on the comparison of a genome with undefined genes to one or several well characterized genomes in order to tentatively determine genes through sequence homology. Biochemical techniques can then be used to confirm the identity of the putative genes identified by homology. However, thermostable enzymes tend to have extensive modifications to their peptide sequences, and thus their nucleotide sequences as well, in order to preserve structure and function at elevated temperatures. Analogous proteins with similar functions in both mesophiles and thermophiles have significantly different nucleotide sequences, making homology-based searches difficult, if not impossible (Figure 4).

Biochemical techniques suitable for identifying copper binding proteins would involve either immobilized metal affinity chromatography or labeled copper. Immobilized copper metal affinity chromatography would involve the tethering of copper ions to a column and allowing copper-binding proteins to interact with the immobilized copper ions. However, attaching copper to a tether may significantly impede the ability of a copper-binding protein to incorporate the copper ion, particularly if the copper-binding site is located within a globular protein (86). Labeled copper, either a radioactive isotope of copper or a copper ion bound to a fluorescent marker, poses another set of technical limitations.

Copper-binding proteins could be identified by the growth of *T. thermophilus* in a media where the only source of copper is either a radioactive copper isotope or fluorescent-labeled copper, then assaying all cell proteins for presence of the label. However, given that the longest

lived radioactive isotope of copper (^{67}Cu) has a half life of approximately 67 hours, and that any fluorescent label is significantly larger than a copper ion, there are severe technical limitations to using labeled copper.

Genetic techniques to identify novel genes tend to involve large-scale knockout mutagenesis until the desired mutant phenotype is observed. Once a mutant has been identified, the location of the mutation on the genome is identified and the mutated gene characterized for the function of interest. However, in order to identify genes important in copper transport and loading in *T. thermophilus*, they would have to be disabled. This would be a fatal mutation, owing to the critical importance of the heme-copper sites in the movement of electrons through CcO.

Experimental Approach. We propose to identify genes and gene products involved in copper-transport and the loading of copper into the *T. thermophilus* CcO Cu_A and Cu_B sites by identifying loss-of-function mutations of aerobic respiratory pathways and characterizing the mutation. Furthermore, we propose to overcome the previously discussed problems with using genetic techniques in this system by two methods: 1) The use of the previously characterized nitrate reducing facultative anaerobic strains NAR1 and HB27c of *T. thermophilus* and 2) engineering a facultative anaerobic derivative of *T. thermophilus*.

NAR1 and HB27c strains of *T. thermophilus* have previously been tentatively identified to be capable of anaerobic respiration in the presence of nitrate through an encoded nitrate reductase (15). We can utilize this strain to study *T. thermophilus* respiratory chain proteins by knock-out mutagenesis, selecting for mutants that cannot survive under aerobic conditions while being capable of growth under anaerobic conditions in the presence of nitrate.

Alternatively, we can engineer a facultative anaerobic derivative of the obligate aerobic strain *T. thermophilus* HB27. We propose two methods: 1) engineering *T. thermophilus* HB27 to express a recombinant lactate dehydrogenase (*ldh*) and 2) using a metagenomic library obtained from anaerobic thermophilic digester communities (Figure 5). In the first method, we would increase the expression of either a foreign or a native *ldh* gene in order to increase the levels of pyruvate fermentation with the hopes of producing sufficient energy to maintain anaerobic growth. We selected *Thermotoga maritima,* an obligately anaerobic hyperthermophile as the source of the foreign *ldh* (ldh_{Tma}), reasoning that ldh_{Tma} would already be adapted for higher activity under anaerobic conditions. We also chose to attempt to increase the level of expression of *T. thermophilus* HB27's native *ldh* (ldh_{Tth}), as ldh_{Tth} would already be adapted to production in *T. thermophilus* HB27. By increasing the level of lactate fermentation, we hope to sustain anaerobic growth. In the second method, we would transform *T. thermophilus* HB27 with the metagenomic library obtained from anaerobic thermophilic digester communities. Ideally, the metagenomic library will encode an anaerobic protein or pathway whose activity will be sufficient to permit anaerobic growth of *T. thermophilus* HB27. This will allow us to overcome the limitations of genetic techniques, enabling the identification of copper chaperones through knock-out mutagenesis.

Nutritional Physiology. We also propose to explore the nutritional physiology of *T. thermophilus* HB27 in an effort to confirm their ability to metabolize sugars, and determine appropriate modifications to media composition or gene expression levels in order to increase glycolytic flux. With increased glycolytic flux, pyruvate fermentation may be able to provide enough energy for anaerobic growth.

Materials and Methods

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani Broth or in Super Optimal broth with Catabolite repression (SOC). *E. coli* TOP10 and NEB5-α were used primarily in the cloning experiments, and transformants selected on LB plates supplemented with kanamycin (30 or 50 μg/mL), ampicillin (100 μg/mL), or hygromycin (50 μg/mL). *T. thermophilus* HB27 was primarily cultured on either rich or minimal media. Rich media: T. Oshima's Thermus broth and Thermus broth. Minimal media: glucose minimal media (adapted from *E. coli* glucose minimal media), yeast nitrogenous base minimal media, and Thermus minimal media. *T. thermophilus* HB27c and NAR1 were cultured on Thermus broth supplemented with 20 mM or 40 mM KNO_3 (Fisher Scientific). For the purpose of plasmid selection and retention, growth media was supplemented with kanamycin (30 or 50 μg/mL), ampicillin (100 μg/mL), or hygromycin (50 μg/mL). For the purpose of nutritional physiology or growth inhibition studies, growth media was supplemented with various amounts of cuprizone (Fluka), KCN (Sigma), tryptone (Fisher Scientific), yeast extract (Fisher Scientific), D-(+)-glucose (Sigma), and D-(+)-maltose (Sigma). The detailed characteristics of the plasmids pYK189, pMH18, and Tsp3 are given in Table 1. The pedigree of these plasmids is detailed in Wayne, J. and Xu, S.-Y., 1997 (107).

Bacterial Media. LB broth: 10.0 g/L tryptone (Fisher Scientific), 5.0 g/L yeast extract (Fisher Scientific), and 5.0 g/L sodium chloride (Fisher Scientific). 15.0 g/L granulated agar (Difco) added for plates. SOC media: 20 g/L tryptone, 5.0 g/L yeast extract, 5.0 g/L NaCl, 2.5 mM KCl (Fisher Scientific), 10 mM $MgCl_2$ (J.T. Baker Chemicals), 10 mM $MgSO_4$ (Sigma), 20 mM D-(+)-glucose (Sigma), adjusted to pH 7.0. T. Oshima's Thermus medium (70): 5 g/L tryptone, 4 g/L yeast extract, and 2 g/L NaCl. 30 g/L granulated agar added for plates. Thermus

broth (16): 8 g/L tryptone, 4 g/L yeast extract, 3 g/L NaCl, adjusted to pH 7.5. 15 g/L granulated agar added for plates. Minimal media: 6.25 g/L Na_2HPO_4 (Sigma), 0.75 g/L KH_2PO_4 (Sigma), 2 g/L NaCl, 1 mL/L A5 trace metals mix, 0.2 g/L $MgSO_4$ (Sigma), and 1 g/L $(NH_4)_2SO_4$ (Sigma), supplemented with D-(+)-glucose or D-(+)-maltose as required. Yeast nitrogen base supplemented minimal media: 1.7 g Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco), 2 g/L tryptone, 6.25 g/L Na_2HPO_4, 0.75 g/L KH_2PO_4, 2 g/L NaCl, 1 g/L $(NH_4)_2SO_4$, supplemented with 1 mL/L A5 trace metals mix and D-(+)-glucose or D-(+)-maltose as required. Defined Thermus minimal media (SOURCE): 1.17 mg/L $FeC_6H_5O_7 \cdot 5H_2O$ (Fisher Scientific), 1.56 mg/L $MnSO_4 \cdot H_2O$ (Sigma), 0.55 mg/L $ZnSO_4$ (Fisher Scientific), 27.78 µg/L Na_2MoO_4 (Aldrich), 50.0 µg/L $CoCl_2 \cdot 6H_2O$ (Sigma), 250.0 µg/L $CuSO_4 \cdot 5H_2O$ (Baker Scientific), 96.15 mg/L $MgSO_4 \cdot 7H_2O$ (Mallinckrodt), 5.58 mg/L $CaCl_2 \cdot 2H_2O$ (Mallinckrodt), 73.08 mg/L KCl (Fisher Scientific), 109.62 mg/L Na_2SO_4 (Fisher Scientific), 509.62 mg/L NaCl (Fisher Scientific), 590.38 mg/L NH_4Cl (Sigma), 4.65 g/L Trizma base (Sigma), 0.64 g/L $K_2HPO_4 \cdot 3H_2O$ (Fisher Scientific), 0.3 g/L KH_2PO_4 (Fisher Scientific), 8 µg/L biotin (Sigma), 15 mg/L EGTA (Sigma), 42 mg/L $NaHCO_3$ (Fisher Scientific), 111.11 µL/L H_2SO_4 (Sigma), adjusted to pH 7.7 and supplemented with D-(+)-glucose, D-(+)-maltose, cuprizone, and/or KCN as required. All media supplemented as required with the following antibiotics for plasmid retention selection: kanamycin (Sigma), 50 µg/mL or 30 µg/mL; and hygromycin (Sigma), 50 µg/mL.

Bacterial growth conditions. *E. coli* strains were cultured at 37ºC. *T. thermophilus* strains were cultured at 70ºC. Aerobic growth was achieved by vigorous agitation (~250 rpm) of liquid cultures in orbital incubator shakers. Liquid cultures were started using 1% inoculum from overnight stationary phase cultures. Anaerobic conditions for liquid cultures of *T.*

thermophilus strains was achieved by growth in sealed 10mL screw cap tubes filled completely with Thermus Broth supplemented with 20mM to 40mM KNO_3 (Fisher Scientific) and incubated at 70ºC. Mineral oil was also used to eliminate oxygen transfer to medium. Anaerobic conditions for solid media cultures of *T. thermophilus* strains accomplished by incubation of cultures using the BBL GasPak Plus Anaerobic System Envelopes with Palladium Catalyst (Becton, Dickinson, and Company).

Preparation of glycerol stocks of bacterial strains for long-term storage. A 500 µL aliquot of overnight *E. coli and T. thermophilus* cultures grown in the appropriate media was mixed with 500 µL of 40% glycerol in a cryovial and stored at -70ºC.

DNA Manipulation. The transformation of *E. coli* cells was performed according to supplied heat-shock protocols for chemically competent cells. The transformation of *T. thermophilus* was performed in accordance with previously established protocols (49). PCR amplification of various products was performed with the Taq PCR Master Mix kit (QIAGEN) and supplied instructions. The various conditions for PCR amplification of specific products are detailed in Table 2. Plasmids were extracted from stationary phase cells by alkaline lysis and ethanol precipitation. Restriction enzymes (NEB BioLabs), T4 DNA ligase (NEB BioLabs), and SuperScript II reverse transcriptase (Invitrogen) used as recommended by manufacturers. Gel electrophoretic analysis performed on 1% analytical agarose (Sigma) gels at 100V. Gel extraction of nucleic acids from preparative agarose (Bio-Rad) gels performed with the QIAEXII Gel Extraction Kit (QIAGEN) in accordance with the supplied protocol. *T. thermophilus* chromosomal DNA isolated using DNAzol Direct (Molecular Research Center).

Construction of pYK189::*ldh*$_{\mathbf{Tma}}$. The wild type *T. maritima ldh* gene (ldh_{Tma}), coding for *T. maritima* D-lactate dehydrogenase, was amplified by PCR from *T. maritima* chromosomal

DNA with the oligonucleotide primers 24 and 25 (Table 3). The ldh_{Tma}-containing EcoRI-HindIII fragment was gel purified, extracted, and cloned into the corresponding restriction sites of pYK189 to produce pYK189::ldh_{Tma}. Selection for the retention of the plasmid in *E. coli* NEB5-α accomplished by growth on LB media supplemented with ampicillin (100 μg/mL). Selection for the retention of the plasmid in *T. thermophilus* HB27 accomplished by growth on T. Oshima's Thermus media or Thermus broth supplemented with kanamycin (50 μg/mL).

Construction of pMH18::ldh_{Tth}. The wild type *T. thermophilus* HB27 *ldh* gene (ldh_{Tth}), coding for *T. thermophilus* HB27 D-lactate dehydrogenase, was amplified by PCR from *T. thermophilus* chromosomal DNA with the oligonucleotide primers 94 and 95 (Table 3). The ldh_{Tth}-containing PstI-EcoRI fragment was purified and cloned into the corresponding restriction sites of pMH18 to produce pMH18::ldh_{Tth}. Selection for the retention of the plasmid in *E. coli* TOP10 and *T. thermophilus* accomplished by growth on LB media or Thermus broth, respectively, supplemented with hygromycin (50μg/mL).

Construction of thermophilic digester DNA libraries. Metagenomic libraries were derived from DNA extracted from anaerobic thermophilic digester communities as previously detailed (34). 1 kb to 10 kb fragments of anaerobic thermophilic digester metagenomic DNA were obtained by partial digest of metagenomic library DNA with the restriction enzymes EcoRI or PstI. These fragments were cloned into the corresponding restriction sites of pMH18 to generate EcoRI or PstI restricted metagenomic libraries. Selection for the retention of this plasmid was accomplished by growth on Thermus broth supplemented with hygromycin (50 μg/mL).

Construction of *malE1* knockout constructs. A truncated internal fragment of the *T. thermophilus* HB27 *malE1* gene encoding trehalose/maltose-binding activity with flanking 5'

and 3' EcoRI restriction sites was amplified by PCR from *T. thermophilus* HB27 chromosomal DNA using the oligonucleotide primers 138 and 139 (Table 3). This fragment was then ligated to the EcoRI restricted fragment of Tsp3 encoding thermostable kanamycin nucleotidyltransferase activity. Single crossover *malE1*::Km^R recombinants were selected for on Thermus broth supplemented with kanamycin (30 μg/mL).

Construction of pMH18::*malE1*. The wild type *T. thermophilus* HB27 *malE1* gene, coding for *T. thermophilus* HB27 trehalose/maltose-binding function, was amplified by PCR from *T. thermophilus* chromosomal DNA with the oligonucleotide primers 141 and 142 (Table 3). The *malE1*-containing EcoRI-PstI fragment was purified and cloned into the corresponding restriction sites of pMH18 to produce pMH18::*malE1*. Selection for the retention of the plasmid in *T. thermophilus* was accomplished by growth on Thermus broth, supplemented with hygromycin (50 μg/mL).

Bacterial growth studies. The growth kinetics of bacterial cell cultures was investigated by the periodic measurement of cell culture densities. Cell densities were determined by the measurement of optical density at 600nm in a Beckman-Coulter DU 640B spectrophotometer. Growth curves were obtained by the plotting of cell culture optical density against time. Several replicates were performed for every growth condition.

***T. thermophilus* HB27 cuprizone inhibition studies.** Copper replacement studies were done by culturing *T. thermophilus* HB27 was cultured in defined Thermus minimal medium supplemented with 1 μM, 10 μM, 20 μM, 50 μM, 100 μM, 500 μM, 1000 μM, 1500 μM, or 2000 μM cuprizone. The effects of varying cuprizone concentration on the growth of *T. thermophilus* HB27 were determined by the measurement of cell culture optical density at 600nm and pH changes in the media following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 KCN inhibition studies.** *T. thermophilus* HB27 was cultured in Thermus broth supplemented with 10 μM, 20 μM, 40 μM, 80 μM, 100 μM, 160 μM, 200 μM, 400 μM, 600 μM, 800 μM, or 1000 μM KCN. The effects of varying KCN concentration on the growth of *T. thermophilus* HB27 were determined by the measurement of cell culture optical density at 600nm following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 tryptone supplementation studies.** *T. thermophilus* HB27 was cultured in Thermus broth supplemented with 8 g/L, 10 g/L, 15 g/L, or 20 g/L tryptone. The effects of varying tryptone amounts on the growth of *T. thermophilus* HB27 were determined by the measurement of cell culture optical density at 600nm following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 pyruvate supplementation studies.** *T. thermophilus* HB27 was cultured in Thermus broth supplemented with 0.5%, 1.0%, or 2.0% (wt/vol) pyruvate as required. Media was also supplemented with 10% yeast extract as required. The effects of varying pyruvate concentrations on the growth of *T. thermophilus* HB27 were determined by the measurement of cell culture optical density at 600nm at periodic intervals or following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 glucose supplementation studies.** *T. thermophilus* HB27 was cultured in Thermus broth, minimal media, yeast nitrogen base supplemented minimal media or defined Thermus minimal media supplemented with 0.3%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 5.0%, or 10.0% (wt/vol) of D-(+)-glucose as required. Media was supplemented with 10% yeast extract as required. The effects of varying glucose concentrations on the growth of *T. thermophilus* HB27 were determined by the measurement of cell culture optical density at 600nm at periodic intervals or following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 maltose supplementation studies.** *T. thermophilus* HB27 was cultured in Thermus broth, minimal media, yeast nitrogen base supplemented minimal media or defined Thermus minimal media supplemented with 0.3%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 5.0%, or 10.0% (wt/vol) of D-(+)-maltose as required. Media was supplemented with 10% yeast extract as required. The effects of varying glucose concentrations on the growth of *T. thermophilus* HB27 were determined by the measurement of cell culture optical density at 600nm at periodic intervals or following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 Tris buffer effect studies.** *T. thermophilus* HB27 was cultured in Thermus broth supplemented with 25 mM, 50 mM, or 100 mM Tris buffer as required. Buffer effectiveness was determined by the measurement of cell culture optical density at 600nm and media pH following overnight growth at 70ºC under high aeration.

***T. thermophilus* HB27 HEPES buffer effect studies.** *T. thermophilus* HB27 was cultured in Thermus broth supplemented with 25 mM, 50 mM, or 100 mM HEPES buffer as required. Buffer effectiveness was determined by the measurement of cell culture optical density at 600nm and media pH following overnight growth at 70ºC under high aeration.

RNA manipulation. For RNA experiments, all glassware and plasticware was soaked in 100 mM NaOH for at least 3 hr prior to use and all solutions used were autoclaved for two 30 minute cycles to ensure inactivation of any RNase present. Whole cell RNA extraction from log phase bacterial cultures was done using RNeasy Midi Kits (QIAGEN) according to manufacturer directions. Gel electrophoretic analysis of RNA samples was carried out on 2% analytical agarose gels. cDNA of *T. thermophilus* HB27 *malE1* mRNA transcripts was synthesized using SuperScript II reverse transcriptase (Invitrogen) according to manufacturer instructions. Quantification of mRNA transcript concentration accomplished using the Quantity One software

(Bio-Rad) to analyze *T. thermophilus* HB27 *malE1* cDNA band intensities following gel electrophoretic analysis.

Knock-out mutagenesis of *T. thermophilus* HB27 *malE1*. Knockout mutagenesis of *T. thermophilus* HB27 *malE1* was accomplished by transformation of *T. thermophilus* HB27 with the *malE1* knockout construct. Mutants were selected on Thermus broth supplemented with 30 µg/mL of kanamycin.

Complementation of *T. thermophilus* HB27Δ*malE1*. Complementation of the mutant *T. thermophilus* HB27Δ*malE1* was accomplished by transformation of the mutant with pMH18::*malE1*. Transformants were selected on Thermus broth supplemented with 50 µg/mL of hygromycin. Effects of complementation determined by the comparison of optical densities of *T. thermophilus* HB27 and *T. thermophilus* HB27Δ*malE1*/pMH18::*malE1* at 600nm following overnight growth of cultures on defined Thermus minimal media supplemented with 1% (wt/vol) maltose at 70ºC and vigorous aeration.

Lactate dehydrogenase assays. *T. thermophilus* HB27 carrying pYK189 or pYK189::ldh_{Tma} was grown to mid-log phase and the cells were harvested by centrifugation. Cells were washed in 0.1 M phosphate buffer, pH 7.0, resuspend in 0.1 M phosphate buffer and cell densities were adjusted to 1 A_{450}/mL. We assume 1 A_{450} cells is equivalent to 350 µg protein/mL (unpublished results). Cell extracts were prepared by brief sonication of cell suspensions with intermittent cooling on ice. Varying amounts of extract were added to reaction tubes containing 10 mM pyruvate in 0.1 M phosphate buffer and tubes were equilibrated at 70C. After equilibration, NADH was added to tubes to final concentration of 0.1 mM, tubes were rapidly mixed and reactions were carried out at 70C for 1 minute. Total reaction volume was 0.8

mL. Reactions were stopped with the addition of 9.0 M urea. Activity values were calculated as μmoL NADH oxidized · min^{-1} · μg $protein^{-1}$. All reactions were performed at least twice.

Results

Growth of Nitrate Reducing *T. thermophilus* strains. *T. thermophilus* HB27c and NAR1 strains, although previously demonstrated to exhibit anaerobic growth on media supplemented with nitrate (15, 16), did not display significant growth increases over the obligately aerobic *T. thermophilus* HB27 strain under anaerobic conditions in Thermus media supplemented with nitrate. Little growth was observed under purely anaerobic conditions (Figure 7) and insignificant growth increases were observed under microaerophilic conditions (Figure 8). Furthermore, only insignificant differences in growth were observed between overlaying the culture with mineral oil to preserve anaerobic conditions or leaving a small headspace (Figure 9).

Expression of *T. maritima ldh* in *T. thermophilus* HB27. A DNA fragment encoding *Thermotoga maritima* lactate dehydrogenase (ldh_{Tma}), with added 5'-EcoRI and 3'-HindIII restriction sites was successfully amplified from *Thermotoga maritima* genomic DNA (Figure 10). The plasmid vector pYK189 is linearized following EcoRI or HindIII restriction endonuclease digests (Figure 11). However, linearized pYK189 bands were observed to be larger than the 9.1 kb specified by previous work. Double restriction endonuclease digests of pYK189 with both EcoRI and HindIII resulted in a smaller linear vector fragment than single restriction endonuclease digests with EcoRI or HindIII, as well as the appearance of a 3 kb fragments (Figure 12). This identified our vector as not pYK189, but a fusion of the plasmid vectors pYK189 and pUC19.

We demonstrated that plasmid vector pUC19-pYK189 can be transformed into *T. thermophilus* HB27 by successful growth of *T. thermophilus* HB27 on media supplemented with

kanamycin. We further confirmed the dependence of thermostable kanamycin resistance on transformation with pUC19-pYK189 by extracting the plasmid vector (Figure 13).

Successful ligation of the plasmid vector with ldh_{Tma} was demonstrated by colony PCR amplification of ldh_{Tma} from pYK189::ldh_{Tma} transformed *T. thermophilus* HB27 cells demonstrating kanamycin resistance (Figure 14). Lactate dehydrogenase assays demonstrated LDH_{Tma} activity (Figure 15). Attempts to culture *T. thermophilus* HB27/ pYK189:ldh_{Tma} anaerobically were not successful.

Construction of pMH18::ldh_{Tth}. Amplification of a DNA fragment encoding the *Thermus thermophilus* HB27 lactate dehydrogenase (ldh_{Tth}) from *T. thermophilus* HB27 genomic DNA was attempted. Amplification of ldh_{Tth} from phenol/chloroform extracted *T. thermophilus* HB27 genomic DNA was not successful, while amplification of ldh_{Tth} from genomic DNA extracted by DNAzol treatment was successful (Figure 16).

The restriction endonucleases PstI, XbaI, and EcoRI were determined to cut a single site on the plasmid vector pMH18 (Figure 17). pMH18 was further determined to confer thermostable hygromycin resistance when transformed into *T. thermophilus* HB27. Successful ligation of the plasmid vector demonstrated by the presence of linearized pMH18 and ldh_{Tth} bands following PstI and EcoRI restriction endonuclease digestion of plamids extracted from *E. coli* DH5α cells transformed with pMH18::ldh_{Tth} and demonstrating hygromycin resistance (Figure 18).

Increasing ldh_{Tth} expression *T. thermophilus* HB27 genomic DNA libraries. Of the three restriction endonucleases determined to cut at a single site on pMH18, only overnight PstI restriction digests resulted in many fragments of *T. thermophilus* HB27 genomic DNA approximately 2 to 10 kb in size. XbaI and EcoRI restriction digests primarily resulted in *T.*

thermophilus HB27 genomic DNA fragments greater than 10 kb in size (Figure 19). PstI-digested *T. thermophilus* HB27 genomic DNA fragments were successfully ligated into pMH18::ldh_{Tth} by means of the PstI site upstream of the insert. Extraction of plasmids from *T. thermophilus* HB27/pMH18::ldh_{Tth}+metagenomic-DNA-fragments and subsequent digestion with PstI demonstrated inserts of varying sizes (Figure 20)

Construction of pMH18::metagenomic DNA library and screens for anaerobic growth. Overnight PstI and EcoRI restriction digests of thermophilic anaerobic digester community metagenomic DNA resulted in larger quantities of 2 to 10 kb DNA fragments than XbaI restriction digests (Figure 19). Through EcoRI and PstI restriction enzyme digestion titrations with the metagenomic DNA sample, we determined that undiluted enzyme obtained the largest range of DNA fragments in the 2 to 10 kb range. In particular, metagenomic DNA fragments less than 10 kb in size were only observed with undiluted (20,000 units/mL) EcoRI, while more high molecular-weight products were observed in increasingly diluted PstI digestions until few metagenomic DNA fragments below 10 kb in size are observed with a four-fold dilution (5,000 units/mL) of the stock enzyme (Figure 21). Activity units are defined as the amount of enzyme required to digest 1 μg of λ DNA in 1 hour at 37ºC in a total reaction volume of 50 μL. No difference in DNA fragment sizes between 40 minute and 70 minute PstI digestions and between 75 minute and 120 minute EcoRI digestions of metagenomic DNA samples was observed (Figure 22). 2 to 10kB DNA fragments of PstI- and EcoRI- digested metagenomic DNA samples were successfully ligated into pMH18 by means of the PstI or EcoRI site upstream of the insert (Figure 23). *T. thermophilus* HB27 was successfully transformed with pMH18::metagenomic-DNA-fragments, as observed by the acquisition of hygromycin resistance following transformation and culture under aerobic conditions.

Extraction of plasmids from *T. thermophilus* HB27/pMH18::metagenomic-DNA-fragments and subsequent digestion with PstI demonstrated inserts of varying sizes, although most inserts were of lower molecular weight (Figure 24). However, repeated attempts to culture *T. thermophilus* HB27/pMH18::metagenomic-DNA-fragments in solid media under anaerobic conditions were not successful.

Construction of the *T. thermophilus* HB27 *malE1* knockout constructs. A truncated fragment of the *T. thermophilus* HB27 *malE1* gene was successfully amplified with the 138/139 primer pair (Figure 25). This truncated fragment was then ligated to the thermostable kanamycin resistance marker derived from EcoRI restriction endonuclease digested Tsp3 plasmid vectors to form the *malE1* knockout construct.

Amplification of *malE1* for future complementation studies. *T. thermophilus* HB27 *malE1* was successfully amplified from *T. thermophilus* genomic DNA (Figure 26).

***T. thermophilus* HB27 growth inhibition studies.** Severe inhibition of growth of *T. thermophilus* HB27 cultured in defined Thermus minimal media supplemented with varying concentrations of the copper chelator cuprizone occurs between 20 μM and 100 μM of cuprizone (Figure 27a Cuprizone inhibition). Measurements of media pH following overnight growth demonstrated minor decreases in pH due to cell growth (Figure 27b).

KCN inhibition of *T. thermophilus* HB27 growth was observed to occur between 400 μM and 600 μM of KCN when cultured in Thermus medium supplemented with varying concentrations of KCN (Figure 28).

Media Optimization. Of the various media types, culture of *T. thermophilus* HB27 was the most optimal in Thermus medium (data not shown). For physiology studies requiring minimal medium, minimal medium and yeast nitrogen base supplemented minimal medium were

not ideal, while defined Thermus minimal media proved to be the most ideal minimal media (Figure 29). In particular, defined Thermus minimal medium offered several advantages over other tested minimal medium. While growth of *T. thermophilus* HB27 in minimal medium supplemented with yeast extract proved to have similar, if not better growth yields than growth in defined Thermus minimal medium, defined Thermus minimal medium allowed precise control of the exact composition of the minimal medium. Buffering of Thermus media with varying concentrations of Tris or HEPES buffer proved to have little effect on *T. thermophilus* HB27 growth (Figure 30).

***T. thermophilus* HB27 nutritional physiology – Tryptone.** Minor enhancement of growth was observed with increasing supplementary concentrations of tryptone following 13 hours of growth in Thermus medium, although after 27 hours little difference was noticeable (Figure 31).

***T. thermophilus* HB27 nutritional physiology – Pyruvate.** Insignificant increases in *T. thermophilus* HB27 culture cell density was observed with increases in pyruvate from 0% to 1% (wt/vol). Severe growth inhibition was observed at 2% pyruvate (Figure 32).

***T. thermophilus* HB27 nutritional physiology – Glucose.** Steady decreases in cell density following overnight growth in Thermus media supplemented with glucose are correlated with increasing amounts of glucose in the medium. Thermus medium can be supplemented with up to 1.0% glucose before significant growth inhibition occurs (Figure 33). Increasing growth in defined Thermus minimal medium was shown to be proportional to increasing glucose concentration. However, slower rates of growth and lower total cell densities are observed when *T. thermophilus* HB27 is cultured in defined Thermus minimal medium compared to culture in Thermus medium (Figure 34).

***T. thermophilus* HB27 nutritional physiology – Maltose.** Slight increases in cell density following overnight growth in Thermus medium supplemented with maltose are correlated with increasing amounts of maltose in the medium (Figure 35). Supplementation of defined Thermus minimal medium with 1% (wt/vol) maltose demonstrates the best growth characteristics (Figure 36). Rates of growth and total cell densities of *T. thermophilus* HB27 are lower in defined Thermus minimal medium supplemented with maltose than if supplemented with glucose or if cultured in unsupplemented Thermus medium (Figure 34).

Knockout of *T. thermophilus* HB27 *malE1*. Transformation of *T. thermophilus* HB27 with the *malE1* knockout construct results in the acquisition of thermostable kanamycin resistance implying the successful knockout mutation of the *T. thermophilus* HB27 *malE1* gene by Campbell-type integration of the *malE1* knockout construct into the native *T. thermophilus* HB27 *malE1* gene. Following 18 hours of growth on defined Thermus minimal medium supplemented with either 1% glucose or maltose, *T. thermophilus* HB27 cells transformed with the *malE1* knockout construct demonstrated limited growth on medium supplemented with maltose, while showing no differences in growth from wild-type strains on medium supplemented with glucose (Figure 37).

Discussion

Nitrate reducing *T. thermophilus* strains. The nitrate reducing *T. thermophilus* strains NAR1 and HB27c were previously reported to grow anaerobically in media supplemented with nitrate (15, 16). Despite numerous attempts, we were unable to detect any significant anaerobic growth of these strains under a variety of anaerobic conditions (Figure 9). Any observed anaerobic growth by the NAR1 or HB27c *T. thermophilus* strains occurred at approximately the same rates in media regardless of nitrate content (Figure 8). Previous studies report the control of the nitrate respiratory apparatus by an oxygen sensor, preventing the reduction of nitrate under aerobic conditions (15). We initially suspected oxygen contamination of our *T. thermophilus* NAR1 and HB27c cultures; however, anaerobic growth was still not observed when additional precautions, such as overlaying the growth media with mineral oil, were taken to prevent gas exchange of the media with the atmosphere. Oxygen contamination does not appear to account for the inability of *T. thermophilus* NAR1 and HB27c strains to grow anaerobically. Our results suggest that nitrate reductase activity of these strains may serve a dissimilatory reductive function under conditions of suboptimal O_2 concentrations, but that the enzyme complex does not support growth under strictly anaerobic conditions.

It is possible that the conditions reported by Cava et al. 2008 (15) and Cava et al. 2007 (16) suitable for anaerobic growth of NAR1 and HB27c *T. thermophilus* strains are unique to particular reagents or culture conditions culture conditions we were unable to duplicate. Otherwise, it would appear that these "anaerobic" strains require some oxygen present in the media, *i.e.* microaerophilic conditions. Without *T. thermophilus* strains capable of efficient anaerobic growth, copper metalloproteins cannot be identified by genetic knock-out mutagenesis techniques. *T. thermophilus* strains must be engineered to be capable of anaerobic growth.

Recombinant DNA techniques to engineer an anaerobic *T. thermophilus* derivative – Lactate dehydrogenase. Fermentation pathways allow the direct conversion of monosaccharide sugars into energy, in the form of ATP, through relatively simple metabolic pathways. In the case of the lactate dehydrogenase (LDH), pyruvate is converted to lactate and NAD^+ is regenerated, preventing glycolysis from stalling. By supplementing the activity of native *T. thermophilus* HB27 LDH, and subsequently increasing NAD^+ regeneration, glycolysis alone may be capable of supporting fermentative growth of *T. thermophilus* under anaerobic conditions.

Thermotoga maritima lactate dehydrogenase (ldh_{Tma}) was initially chosen due to the extreme thermophilic and fermentative properties of the organism. ldh_{Tma} would theoretically operate at high efficiency under the anaerobic and thermophilic conditions necessary for our study. However, while recombinant ldh_{Tma} activity in *T. thermophilus* HB27/pYK189::ldh_{Tma} was observed (Figure 15), this increased activity was insufficient to support anaerobic growth. Activity of ldh_{Tma} may not be as high as expected due to the lower optimal growth temperature of *T. thermophilus* as compared to the optimal growth temperature of *T. maritima* of approximately 80°C.

Native *T. thermophilus* HB27 lactate dehydrogenase (ldh_{Tth}) was chosen next in the hopes that, being a native protein, optimal activity would be maintained by the cell. By increasing the expression of ldh_{Tth}, either by the means of a previously identified strong native promoter or through strong promoters recovered from libraries of either *T. thermophilus* HB27 genomic DNA or thermophilic anaerobic digester community metagenomic DNA, we hoped to sufficiently boost fermentative pathways to support anaerobic growth. The capacity for anaerobic growth was chosen as the screen for sufficient elevation of fermentative pathways. LDH assays were not performed to confirm increased expression of recombinant *T. thermophilus*

LDH as anaerobic growth would imply sufficiently increased expression of LDH and thus, fermentation rates, to support anaerobic growth. However, no *T. thermophilus* HB27 cells transformed with pMH18::ldh_{Tth}, pMH18::ldh_{Tth}+*T. thermophilus*-genomic-DNA-fragments, or pMH18::ldh_{Tth}+metagenomic-DNA-fragments displayed the capacity for anaerobic growth. We briefly considered the possibility that fermentation products were causing an acidification of the medium and subsequent inhibition of growth; however, buffering of the growth medium with HEPES or Tris buffers did not yield any anaerobic derivatives.

Despite the increased expression of *T. maritima* or *T. thermophilus* HB27 LDH, insufficient rates of fermentation occurred in order to support anaerobic growth. This implies the levels of glycolytic flux to be too low for adequate fermentative processes to occur. As a result, we determined the need to better understand *T. thermophilus* HB27 physiology in the hopes of increasing glycolytic flux to levels sufficient to support anaerobic growth.

Recombinant DNA techniques to engineer an anaerobic *T. thermophilus* derivative – Metagenomic Library DNA. Metagenomic DNA libraries provide an efficient way to sample a wide variety of genes from a diverse group of organisms, not all of which may be fully characterized. This allows the discovery of novel genes or novel combinations of genes that can result in a particular desired activity. In this instance, the metagenomic DNA library is derived from metagenomic DNA samples obtained from a community of anaerobic thermophilic digesters maintained at 55°C for a period of two decades (34). Hypothetically, the derived metagenomic DNA library contains intact genes and pathways, some of which will encode anaerobic fermentation or respiratory elements. Subsequent expression in *T. thermophilus* HB27 of these metagenomic DNA library gene products may be adequate to allow growth under anaerobic conditions.

While the metagenomic DNA library was demonstrated to have been successfully constructed (Figure 23), albeit composed primarily of low molecular-weight inserts, transformation of these libraries into *T. thermophilus* HB27 was insufficient to allow anaerobic growth. Several reasons for this lack of anaerobic growth exist. First, there may have been an inadequate number of unique library constructs encoding intact genes and pathways transformed into *T. thermophilus* HB27. Statistically, given sufficient numbers of unique intact genes transformed into *T. thermophilus* HB27, an anaerobic derivative would have been obtained. However, due to temporal and physical constraints, only so many samples can be processed at a single time, indicating that a more direct method of selecting inserts may be needed. Sequencing the metagenomic DNA library fragments and performing homology-based searches for putative thermostable anaerobic fermentative or respiratory pathways, followed by the expression of these putative genes in *T. thermophilus* HB27 may be more successful than our current approach.

Furthermore, given the age of the metagenomic DNA sample, sufficient degradation may have occurred to the point where recovering intact genes or pathways may be unlikely. New metagenomic DNA samples from thermophilic anaerobic communities could be obtained to ensure age-related degradation of the sample is not a limiting factor.

Finally, given the large fraction of low molecular-weight metagenomic DNA library inserts (Figure 24), few intact genes may be present in the library. This may be result of age-related degradation of the metagenomic DNA sample, or an oversight in the library construction technique that favors the ligation of lower molecular-weight insert sizes over higher molecular-weight insert sizes. Improvements to the library construction technique that would favor the incorporation of higher molecular-weight inserts, and thus increase the likelihood of incorporating intact genes and pathways, could overcome this limitation.

Media studies. We determined that the best growth rates were observed on Thermus medium, while growth on defined Thermus minimal medium resulted in the best growth rates of any of the minimal media tested. Other mediums tried resulted in less growth than the above mentioned (T. Oshima's Thermus medium, minimal medium, minimal medium supplemented with yeast extract, data not shown), or, in the case of yeast nitrogen base supplemented minimal medium, significantly inhibited growth of *T. thermophilus* HB27 under certain conditions, in this case, when the medium was supplemented with glucose.

Growth inhibition studies. The genetic approach to identifying copper metalloproteins involves the inhibition of aerobic respiratory pathways, resulting in an inability to respire aerobically. While this would normally be a fatal mutation in the obligately aerobic *T. thermophilus*, an anaerobic derivative would be able to survive by means of anaerobic fermentative or respiratory pathways. This necessitates the ability to completely disable aerobic respiratory pathways. Two methods of inhibition were determined: 1) copper chelation and 2) inhibition of the cytochrome c oxidase with cyanide.

The importance of copper to *T. thermophilus* HB27 cell growth is demonstrated by the severe inhibition of growth with as little as 100 μM cuprizone (Figure 27a). Cuprizone chelation of copper can be used as a selection method for the inhibition of aerobic respiratory pathways. Cyanide binds to iron centers of cytochrome c oxidase, effectively preventing the movement of electrons, thus disabling the terminal oxidase and causing the electron transport chain to stall. This serves as a method to specifically disable the cytochrome c oxidase and thereby inhibit aerobic respiration. Our work has demonstrated that cuprizone and cyanide should be useful agents for the selection of anaerobic *T. thermophilus* derivatives.

Nutritional physiology studies. We have shown that we can successfully express active recombinant proteins in *T. thermophilus* HB27 (Figure 15). However, as previously discussed, these methods are insufficient for anaerobic growth. The limiting factor may be low rates of monosaccharide metabolism, and subsequently low rates of glycolytic flux. To investigate this possibility, we wish to establish media conditions conducive to fermentative growth, *i.e.* high sugar content, that do not significantly inhibit *T. thermophilus* HB27 growth. To this end, we studied the effects of various supplements.

Since *T. thermophilus* is best suited to growth on peptides (14), we expected increases in growth proportional to increasing amino acid availability, *i.e.* increasing tryptone concentration. However, this was shown not to be the case (Figure 31). This suggests that growth is limited by some factor other than carbon-source availability. Similar studies with pyruvate demonstrated slight increases in total cell yield up to approximately 1-2% pyruvate, whereupon severe inhibition of growth occurs (Figure 32). This suggests that simply increasing the substrate for LDH will not be sufficient for anaerobic fermentation, especially if *T. thermophilus* is sensitive to pyruvate concentration. As monosaccharides are not the preferred carbon source, sugar metabolism may not be very fast or efficient. *T. thermophilus* may be near maximum sugar utilization rates already without additional media supplementation. Further studies are needed to determine the precise mechanism of pyruvate-mediated growth inhibition.

Increasing glycolytic flux will require increasing the availability of monosaccharide substrates. However, increasing the amount of glucose above 3.0% (wt/vol) inhibits both growth rate and final cell density of *T. thermophilus* HB27 in Thermus medium and defined Thermus minimal medium. 0.3% glucose resulted in the fastest growth rates, while 1.0% glucose resulted in lower growth rates but greater total cell yields (Figure 34). The increasing length of the lag

phase correlated with increasing glucose concentration indicates that prior adaptation of the cell to glucose processing must first occur before glucose can be efficiently utilized. This further implies that in order to increase glycolytic flux in *T. thermophilus* HB27 using glucose as the substrate requires decreasing the length of the lag phase. This could possibly be accomplished by identifying genes involved in glucose processing attenuation and increasing their level of activity.

We also investigated growth of *T. thermophilus* HB27 on maltose-containing medium. In these experiments, we found that increasing maltose concentration correlates with increased total cell yields. Even culture in 10% maltose (wt/vol) in Thermus medium shows greater total cell yield than in unsupplemented media (Figure 36). Growth rates are practically independent of maltose concentration. When maltose is the only carbon source in the media, the dependence of *T. thermophilus* HB27 growth on maltose concentration is more apparent. Growth rates are slower than those on glucose in defined Thermus minimal media, as are total cell yields (Figure 34). From these studies, we determined maltose to be the more appropriate of the two sugars to use in media supplementation due to its less negative effects on growth at higher sugar concentrations.

Genes involved in maltose metabolism. Genes associated with maltose transport are either uncharacterized or have been biochemically characterized. We determined a need to examine the role of these genes in maltose metabolism in order to identify genes important in maltose import and processing. Increased expression of these genes may increase maltose metabolism, and subsequently increasing glycolytic flux.

malE1 was identified as a putative maltose/trehalose transporter. Knockout mutagenesis of this gene demonstrated its importance in the ability of *T. thermophilus* HB27 to utilize maltose

(Figure 37). Complementation assays are under way to confirm the role of *malE1* in *T. thermophilus* HB27 maltose metabolism. Various other genes were also identified as being involved in maltose transport; however, time constraints precluded investigation.

Conclusion.

The engineering of any organism to display non-native physiological characteristics is an extremely complicated process. Detailed understanding of the target organisms' physiology, genome, and relevant proteins must exist, as well as a good understanding of the various interactions between these components.

In contradiction with previous work demonstrating the ability of *T. thermophilus* NAR1 and HB27c strains to grown under anaerobic conditions when supplemented with nitrate (14), we have shown that neither strain exhibits significant growth under anaerobic conditions in the presence of nitrate. Furthermore, any observed growth appears to be the result of aerobic respiration using residual oxygen remaining in the growth medium. Anaerobic growth may be achieved under very specific, unreported conditions unique to the experimental methods of previous researchers (15, 16).

The expression of recombinant enzymes involved in anaerobic fermentative and respiratory pathways alone in *T. thermophilus* HB27 proved insufficient for anaerobic growth. The supplementation of the medium with monosaccharide sugars appears to be necessary for increasing glycolytic flux. Our current goal is to complete the physiological characterization of the *T. thermophilus* HB27 maltose utilization apparatus. Following complete characterization of the system, we aim to increase levels of maltose transport and metabolism. Our long term aim is to increase glycolytic flux to levels where anaerobic growth is possible.

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Tables

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant description or genotype[a]	Source or Reference
Eshcerichia coli		
TOP10	*hsdR mcrA lacZ**Δ**M15 endA1 recA1*	Invitrogen
NEB 5-α	*fhuA2 **Δ**(argF-lacZ)U169 phoA glnV44 φ80**Δ**(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England BioLabs
Thermus thermophilus		
HB27	wild-type *T. thermophilus* strain	ATCC BAA-163
HB27c	*nce*	J. Berenguer
NAR1	*nce*	J. Berenguer
Thermotoga maritima		
MSB8	wild type *T. maritima* strain	ATCC 43589
Plasmids		
Tsp3	*Thermus-E. coli* shuttle vector, Ap^R, thermostable Km^R, ColE1 *ori*, P_{taq}, ΔpTT8, 9.6kb	158
pUC19-pYK189	*Thermus-E. coli* shuttle vector, Ap^R, thermostable Km^R, ColE1 *ori*, P_{taq}, ΔpTT8, 11.7kb	158
pMH18	*Thermus-E. coli* shuttle vector, thermostable Hy^R, *lacZ*α, ColE1 *ori*, ΔpTT8, 5.6kb	J. Berenguer

[a] Ap^R, ampicillin resistance; Km^R, kanamycin resistance

Table 2. PCR reaction conditions used in this study.

Product (Primer Pairs)	Annealing Temp.	Extension Time
ldh_{Tma} (24/25)	55ºC	0.75 min.
ldh_{Tth} (94/95)	55ºC	0.75 min.
'malE1' (138/139)	65ºC	2 min.
malE1 (140/141)	40ºC -80ºC	7 min.
malE1 (142/141)	64ºC	7 min.

Table 3. Oligonucleotide primers used in this study.

Name	Sequence (5' - 3') [a]	Comments
24	TTGAATTCCAGTCCAGCACACCAACAAC	*T. maritima ldh* forward primer, EcoRI
25	CCAAGCTTCAAACTTCGAAAGGGGAAAG	*T. maritima ldh* reverse primer, HindIII
94	AACATCTGCAGCGAGGAGAACAGAGATGAAGGTCGGCATCGTGGGA	*T. thermophilus ldh* forward primer, PstI
95	TCAACGGGAAGAATTCGGAGAAGGAGGGCGAGGGCCTCAC	*T. thermophilus ldh* reverse primer, EcoRI
138	AATGAATTCCCCGTATCGTCCAGAACAAC	Truncated *T. thermophilus* HB27 *malE1* forward, EcoRI
139	ACGAATTCCTGTAGGCGGAGACCATGAG	Truncated *T. thermophilus* HB27 *malE1* reverse, EcoRI
140	TAAAGGTACCGCTTTGGGTGTGCTTGAAAC	*T. thermophilus* HB27 *malE1* forward, KpnI
141	ACCTACGACTTGCTGCAGATAAAT	*T. thermophilus* HB27 *malE1* reverse, PstI
142	TAAAGAATTCGCTTTGGGTGTGCTTGAAAC	*T. thermophilus* HB27 *malE1* forward, EcoRI

[a] Underlined bases denote restriction endonuclease recognition site, as indicated under comments.

Figure Legends

Figure 1. Phylogenetic tree based on analysis of small subunit rRNA. Thick lines represent hyperthermophilic lineages. Adapted from (90).

Figure 2. *T. thermophilus* HB8. **A**) Phase-contrast microscope, magnification x1,000. Bar indicates 12 μm. **B**) Electron micrograph, magnification x27,500. Bar indicates 0.5 μm. Adapted from (70).

Figure 3. A) Cytochrome *c* oxidase depicted embedded within the phospholipid bilayer and with bound cytochrome c (blue subunit). Cytochrome c reduces cytochrome c oxidase by transferring electrons through Cu_A to the haem a_3 / Cu_B site of O_2 reduction. Protons are translocated across the membrane and H_2O is generated. **B**) Eukaryotic model of copper loading of cytochrome c oxidase Cu_A and Cu_B sites. Adapted from (8).

Figure 4. Peptide sequence alignment of portions of the cytochrome c oxidases of various species. **A**) The sequence alignment of the thermophilic bacterium *Thermus thermophilus* and *Rhodothermus marinus* demonstrate conservation of peptide sequence. **B**) The sequence alignment of *Saccharomyces cerevisiae* against *Homo sapiens* demonstrates that among non-thermophilic organisms, the peptide sequence of cytochrome c oxidase is also relatively well conserved. **C**) Comparison of all four species reveals that the thermostable cytochrome c oxidase does not demonstrate much sequence similarity with non-thermostable cytochrome c oxidase. Red highlighting denotes identical residues, grey highlighting denotes functionally conserved residues. The Clustal consensus line denotes fully conserved residues (*), strongly conserved residues (:), and weakly conserved residues (.), as defined by the Gonnet PAM250 matrix.

Figure 5. The anaerobic thermophilic community from which the metagenomic libraries were obtained were maintained in 55°C water baths for over 10 years. These communities were fed either dried Napier grass or dried Bermudagrass. DNA was then extracted from mixed liquor samples pooled from both digesters (44).

Figure 6. Graphical description of methods used to engineer anaerobic *T. thermophilus* HB27 derivatives. **A**) Method using metagenomic libraries. **B**) Method using recombinant ldh_{Tth}.

Figure 7. Growth curves of *T. thermophilus* HB27c under anaerobic conditions. Sample conditions as follows: 93% inoculum and 40mM KNO_3 (), 93% inoculum only (), 50% inoculum and 40mM KNO_3 (), 50% inoculum only (), 25% inoculum and 40nM KNO_3 (), and 25% inoculum only ().

Figure 8. Overnight growth of *T. thermophilus* NAR1 under microaerophilic conditions. Insignificant differences in growth observed between NAR1 cultures supplemented with 30mM KNO_3 and unsupplemented cultures. **A**) Cultures inoculated from overnight NAR1 cultures grown in unsupplemented Thermus medium. **B**) Cultures inoculated from overnight NAR1 cultures grown in 30nM KNO_3 supplemented Thermus medium.

Figure 9. Growth curves of *T. thermophilus* NAR1 under anaerobic conditions. Insignificant growth observed when cultures were supplemented with 30mM KNO_3. Culture conditions as follows: Unsupplemented Thermus medium (), unsupplemented Thermus medium overlaid with mineral oil (), 30mM KNO_3 supplemented Thermus medium (), 30mM KNO_3 supplemented Thermus medium overlaid with mineral oil (). All cultures started with 30% inoculum.

Figure 10. Analytical agarose gel electrophoresis of ldh_{Tma} amplified by PCR. PCR amplification of *ldh* from *T.maritima (*Tma) genomic DNA. Lane 1, kb ladder. Lane 2, ~1.4 kb ldh_{Tma} fragment.

Figure 11. Analytical agarose gel electrophoresis of pYK189: Lane 1, kb ladder. Lane 2, pYK189. Lane 3, EcoRI cut pYK189. Lane 4, HindIII cut pYK189.

Figure 12. Analytical agarose gel electrophoresis of restriction endonuclease digested pUC19-pYK189: Lane 1, kb ladder. Lane 2, EcoRI digested pUC19-pYK189. Lane 3, HindIII digested pUC19-pYK189. Lane 4, EcoRI and HindIII double-digested pUC19-pYK189.

Figure 13. Analytical agarose gel electrophoresis of restriction endonuclease digested pYK189 extracted from transformed *T. thermophilus* HB27 cells demonstrating kanamycin resistance. **A**) Lane 1, kb ladder. Lane 2-4, plasmids extracted from three different *T. thermophilus* HB27/pYK189 transformants. **B**) Lane 1, kb ladder. Lane 2, uncut pYK189. Lane 3, EcoRI digested pYK189. Lane 4, HindIII digested pYK189. Lane 5, EcoRI and HindIII double-digested pYK189.

Figure 14. Analytical agarose gel electrophoresis of ldh_{Tma} by colony PCR from pYK189::ldh_{Tma} extracted from transformants. Lane 1, kb ladder. Lane 2, blank control. Lane 3-4, ldh_{Tma} amplicon from extracted pYK189::ldh_{Tma}.

Figure 15. Lactate dehydrogenase assay. Cell-free extracts of aerobically grown cultures of *T. thermophilus* carrying plasmid vector pYK189 or pYK189::ldh_{Tma} were prepared and assayed for lactate dehydrogenase activity as described in Materials and Methods. Activities are given as μmoles NADH oxidized/min/μg protein. Error bars indicate standard deviation. All assays were performed at least twice.

Figure 16. Analytical agarose gel electrophoresis of ldh_{Tth} amplicon. **A**) Amplification attempt from phenol-chloroform extracted genomic DNA. Lane 1, kb ladder. Lane 2-4, ldh_{Tth} amplification attempts from various phenol-chloroform *T. thermophilus* HB27 genomic DNA extract. **B**) Successful amplification of ldh_{Tth} using an annealing temperature of 60°C. Lane 1, kb ladder. Lane 2, ldh_{Tth} amplicon.

Figure 17. Analytical agarose gel electrophoresis of pMH18 following digestion with various restriction endonucleases. Lane 1, kb ladder. Lane 2, uncut pMH18. Lane 3, PstI cut pMH18. Lane 4, XbaI cut pMH18. Lane 5, BamHI cut pMH18. Lane 6, XmnI cut pMH18. Lane 7, KpnI cut pMH18. Lane 8, EcoRI cut pMH18.

Figure 18. Analytical agarose gel electrophoresis of plamids extracted from *E. coli* DH5α cells transformed with pMH18::ldh_{Tth}.. Top row, lane 1, kb ladder. Lane 2, uncut pMH18. Lane 3, PstI/EcoRI cut pMH18. Lane 4, PstI cut pMH18. Lane 5, EcoRI cut pMH18. Top row, lanes 6, 8, 10, and bottom row, lanes 3, 5, 7, 9, 11 uncut

pMH18::ldh_{Tth} from various transformants. Top row, lanes 7, 9, 11, and bottom row, lanes 4, 6, 8, 10, 12, PstI/EcoRI cut pMH18::ldh_{Tth} from various transformants

Figure 19. Analytical agarose gel electrophoresis of various complete restriction endonuclease digests of thermophilic anaerobic digester metagenomic DNA samples and *T. thermophilus* HB27 genomic DNA. Lane 1, kb ladder. Lane 2, uncut thermophilic anaerobic digester metagenomic DNA. Lane 3, uncut *T. thermophilus* HB27 genomic DNA. Lane 4, PstI cut metagenomic DNA. Lane 5, XbaI cut metagenomic DNA. Lane 6, EcoRI cut metagenomic DNA. Lane 7, PstI cut *T. thermophilus* HB27 genomic DNA. Lane 8, XbaI cut *T. thermophilus* HB27 genomic DNA. Lane 9, EcoRI cut *T. thermophilus* HB27 genomic DNA.

Figure 20. Analysis of pMH18::ldh_{Tth}+metagenomic-DNA-library-fragments by agarose gel electrophoresis. Lane 1, kb ladder. Lanes 2, 5, 8, and 11, uncut pMH18::ldh_{Tth}+metagenomic-DNA-library-fragments extracted from various transformants. Lanes 3, 6, 9, and 12, PstI cut pMH18::ldh_{Tth}+metagenomic-DNA-library-fragments extracted from various transformants. Lanes 4, 7, 10, and 13, PstI and EcoRI cut pMH18::ldh_{Tth}+metagenomic-DNA-library-fragments extracted from various transformants.

Figure 21. Analytical agarose gel electrophoresis of thermophilic anaerobic digester metagenomic DNA samples following restriction endonuclease digestion with varying concentrations of restriction endonuclease EcoRI or PstI. Lane 1, kb ladder. Lane 2, uncut thermophilic anaerobic digester metagenomic DNA. Lanes 3-8 are metagenomic DNA samples cut with varying concentrations of EcoRI. Lane 3, cut with 62.5 units/mL EcoRI. Lane 4, cut with 125 units/mL EcoRI. Lane 5, cut with 250 units/mL EcoRI. Lane 6, cut with 500 units/mL EcoRI. Lane 7, cut with 1,000 units/mL EcoRI. Lane 8, cut with 2,000 units/mL EcoRI. Lanes 9-14 are metagenomic DNA samples cut with varying concentrations of PstI. Lane 9, cut with 62.5 units/mL PstI. Lane 10, cut with 125 units/mL PstI. Lane 11, cut with 250 units/mL PstI. Lane 12, cut with 500 units/mL PstI. Lane 13, cut with 1,000 units/mL PstI. Lane 14, cut with 2,000 units/mL PstI.

Figure 22. Analytical agarose gel electrophoresis of partial restriction endonuclease digestions with varying digestion times of thermophilic anaerobic digester metagenomic DNA samples. **A**) PstI digestions of metagenomic

DNA samples. Lane 1, kb ladder. Lane 2, 40 minutes. Lane 3, 50 minutes. Lane 4, 60 minutes. Lane 5, 70 minutes. **B**) EcoRI digestions of metagenomic DNA samples. Lane 1, kb ladder. Lane 2, 75 minutes. Lane 3, 90 minutes. Lane 4, 105 minutes. Lane 5, 120 minutes.

Figure 23. Analytical agarose gel electrophoresis of various steps in the construction of the thermophilic anaerobic digester metagenomic DNA libraries. **A**) Fragments between 1.65kb and 10kb recovered from partial PstI digests of thermophilic anaerobic digester metagenomic DNA samples. Lane 1, kb ladder. Lane 2, PstI digested metagenomic DNA. **B**) Analysis of restriction endonuclease digested plasmid vector pMH18 and metagenomic DNA inserts. Lane 1, kb ladder. Lane 2, PstI cut pMH18. Lane 3, PstI cut metagenomic DNA inserts prepared from recovered fragments. **C**) Analysis of plamids recovered from successful *E. coli* DH5α transformants by PstI restriction endonuclease digestion. Both top and bottom rows: lane 1, kb ladder. Lane 2, uncut pMH18. Lanes 3, 5, 7, and 9, uncut plasmids recovered from various transformants. Lanes 4, 6, 8, and 10, PstI cut plasmids recovered from various transformants.

Figure 24. Agarose gel electrophoretic analysis of thermophilic anaerobic digester metagenomic DNA library by PstI restriction endonuclease digestion. For both top and bottom rows on both agarose gels: Lane 1, kb ladder. Lanes 2, 4, 6, 8, 10, and 12, various uncut metagenomic DNA library plasmids. Lanes 3, 5, 7, 9, 11, and 13, various PstI cut metagenomic DNA library plasmids.

Figure 25. Analytical gel electrophoresis of truncated *T. thermophilus* HB27 *malE1* amplicon. Lane 1, kb ladder. Lane 2, truncated *malE1* amplicon.

Figure 26. Analytical gel electrophoresis of *T. thermophilus* HB27 *malE1* amplicon. Lane 1, kb ladder. Lane 2, *malE1* amplicon.

Figure 27. Growth inhibition of *T. thermophilus* HB27 growth with cuprizone. **A**) Optical density measurements following overnight growth in defined Thermus minimal medium with specified concentrations of cuprizone. **B**)

pH measurements following overnight growth in defined Thermus minimal medium with specified concentrations of cuprizone.

Figure 28. Growth inhibition of *T. thermophilus* HB27 with KCN. Optical density measurements following overnight growth in Thermus medium supplemented with the specified concentrations of KCN.

Figure 29. Growth of *T. thermophilus* HB27 under various minimal media types. **A**) Culture density of *T. thermophilus* HB27 following 13 hours of growth. Culture conditions as follows: minimal media supplemented with glucose (■), minimal media supplemented with glucose and yeast extract (■), and defined Thermus minimal media supplemented with glucose (■). **B**) Culture density of *T. thermophilus* HB27 following 13 hours of growth. Culture conditions the same as in **A**. **C**) Culture density of *T. thermophilus* HB27 following overnight growth in yeast nitrogen base supplemented minimal medium supplemented with either 1% (wt/vol) glucose or maltose.

Figure 30. Growth of *T. thermophilus* HB27 in Thermus medium supplemented with the specified Tris or HEPES buffer concentration. Tris buffered medium (■). HEPES buffered medium (■). **A**) Final cell culture densities following growth at the specified Tris or HEPES concentrations. **B**) Total change in medium pH. Original medium at pH 7.5 (━).

Figure 31. Growth of *T. thermophilus* HB27 in Thermus medium supplemented with additional tryptone. Growth following 13 hours (■). Growth following 27 hours (■).

Figure 32. Final measurements of *T. thermophilus* HB27 cells grown in Thermus medium supplemented with pyruvate.

Figure 33. Growth of *T. thermophilus* HB27 in Thermus medium supplemented with glucose. **A**) Final culture optical density measurements following overnight growth in Thermus medium supplemented with the specified percent (wt/vol) glucose. Error bars indicate standard deviation. **B**) Final culture pH measurements following overnight growth in Thermus medium supplemented with the specified percent (wt/vol) glucose. Original medium at pH 7.5.

Figure 34. Growth of *T. thermophilus* HB27 in either Thermus medium or defined Thermus minimal medium supplemented with varying percent (wt/vol) of glucose and maltose. Growth conditions: Unsupplemented Thermus medium (). Unsupplemented defined Thermus minimal medium (), defined Thermus minimal medium supplemented with 0.3% (wt/vol) glucose (), 1% (wt/vol) glucose (), 0.3% (wt/vol) maltose (), or 1% (wt/vol) maltose (),

Figure 35. Growth of *T. thermophilus* HB27 in Thermus medium supplemented with maltose. **A**) Final culture optical density measurements following overnight growth in Thermus medium supplemented with the specified percent (wt/vol) maltose. **B**) Final culture pH measurements following overnight growth in Thermus medium supplemented with the specified percent (wt/vol) maltose. Original medium at pH 7.5.

Figure 36. Growth of *T. thermophilus* HB27 in Thermus medium supplemented with varying amounts of maltose. Growth conditions: Unsupplemented Thermus medium (). Thermus medium supplemented with 0.5% (wt/vol) maltose (), 1% (wt/vol) maltose (), 1.5% (wt/vol) maltose (), 2% (wt/vol) maltose (), 3% (wt/vol) maltose (), 5% (wt/vol) maltose (), or 10% (wt/vol) maltose ().

Figure 37. Growth of *T. thermophilus* HB27Δ*malE1* and *T. thermophilus* HB27 on defined Thermus minimal medium supplemented with 1% (wt/vol) glucose () or maltose (). Growth in the presence of 30 µg/mL kanamycin only occurs with integration of the *malE1* knockout construct.

Figures

Figure 1

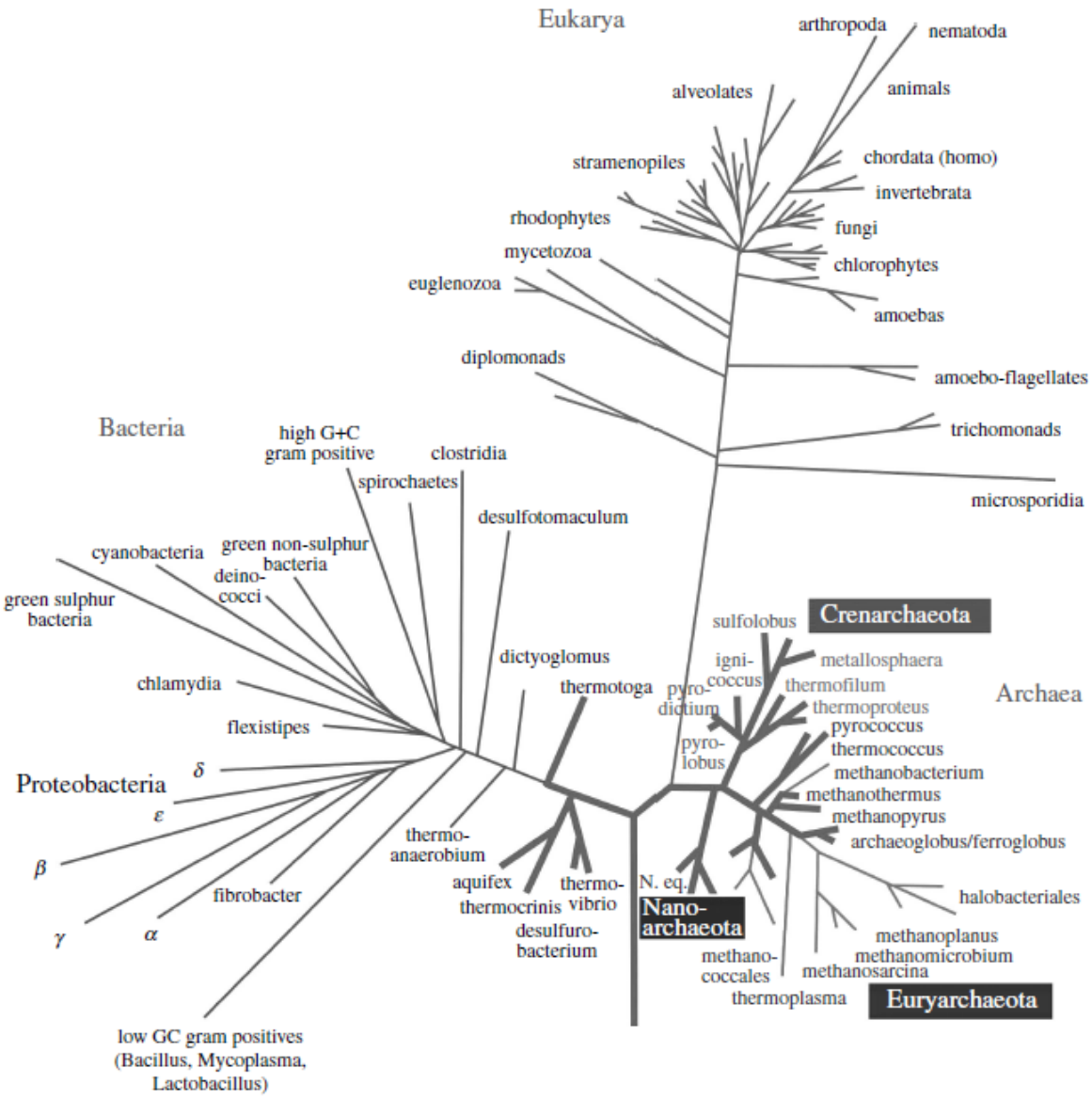

Figure 2

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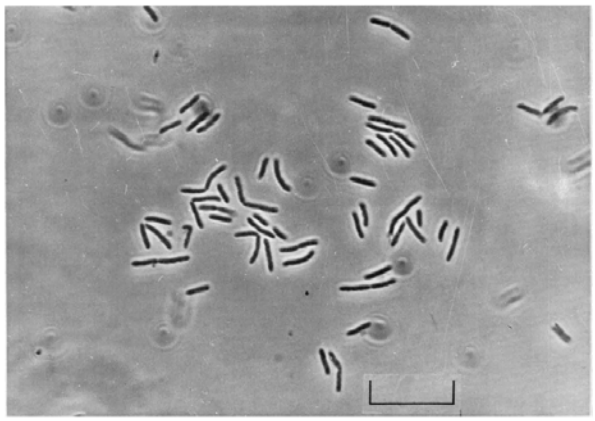

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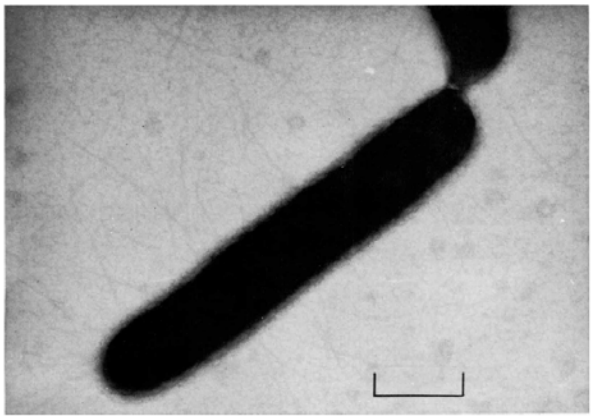
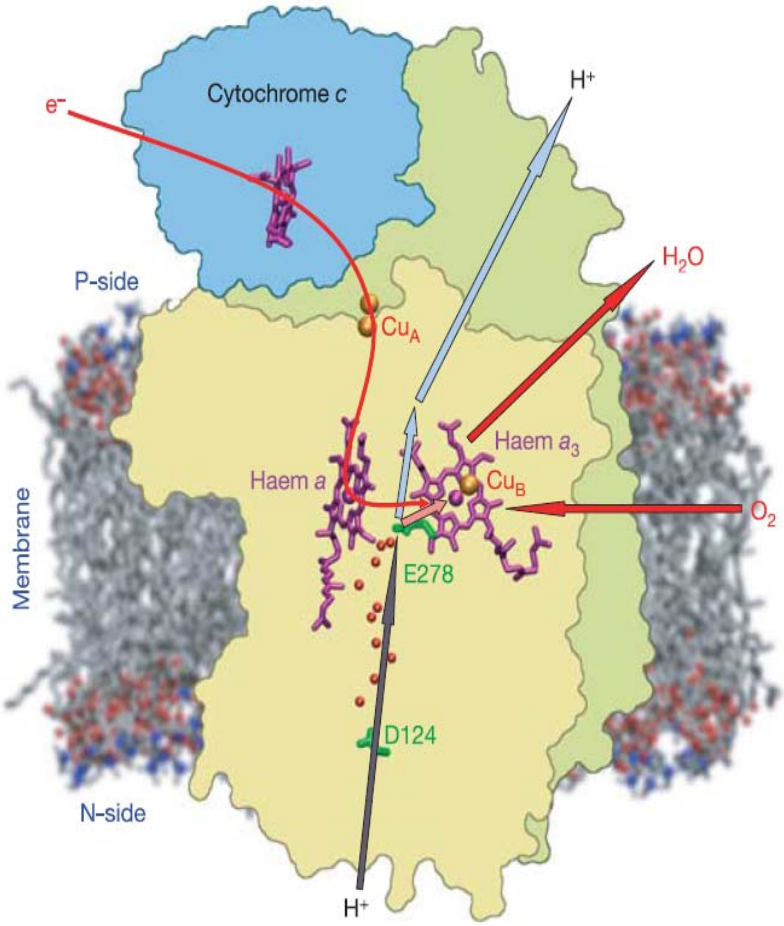


Figure 3

A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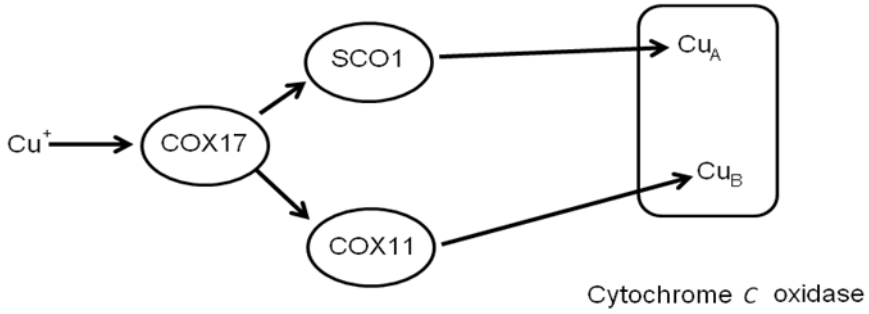
B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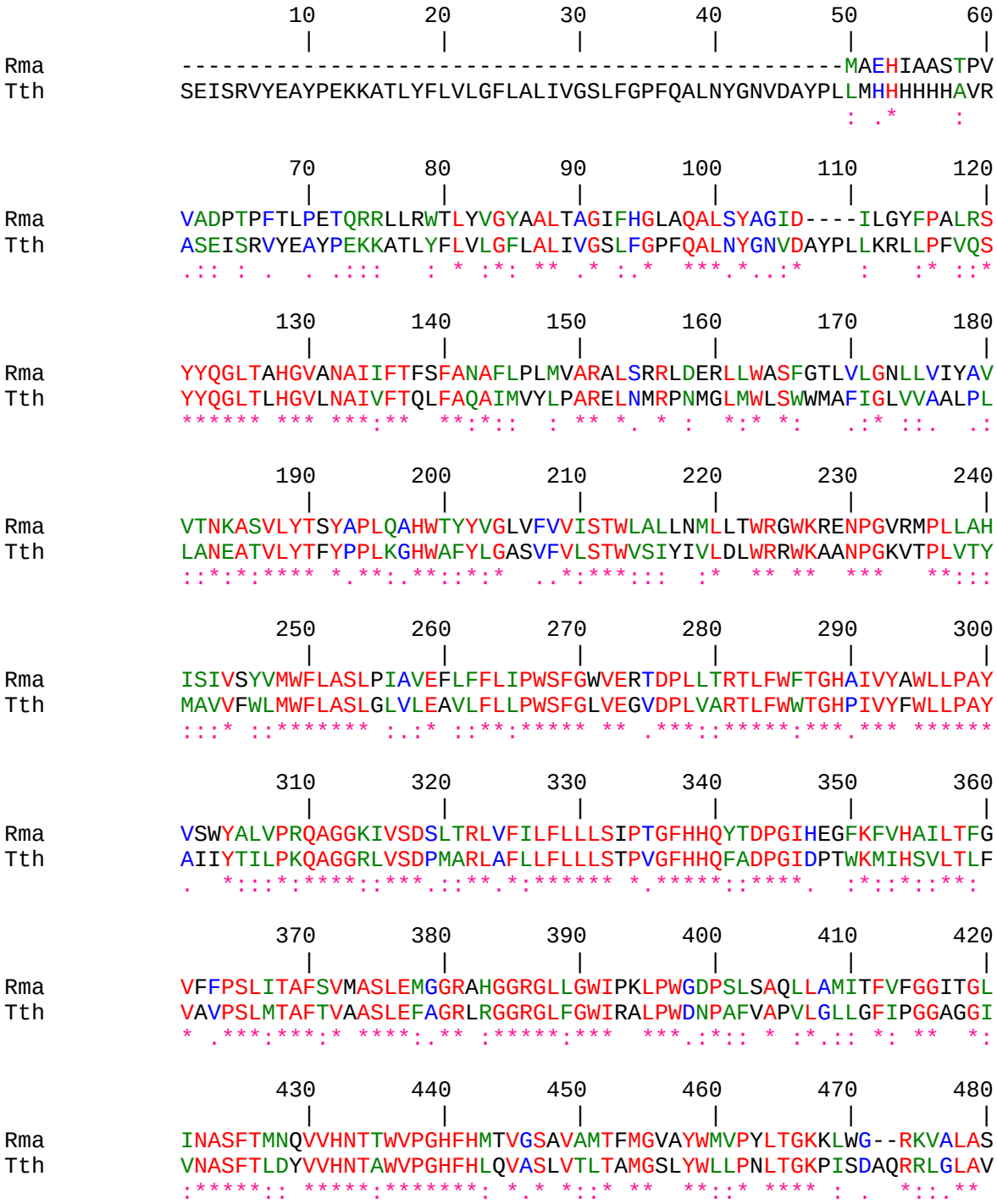
Cu_A
Cu⁺
COX17
Cu_B
COX11
Cytochrome c oxidase

Figure 4A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Rma VADPTPFTLPETQRRLLRWTLYVGYAALTAGIFHGLAQALSYAGID----ILGYFPALRS
Tth ASEISRVYEAYPEKKATLYFLVLGFLALIVGSLFGPFQALNYGNVDAYPLLKRLLPFVQS
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Tth YYQGLTLHGVLNAIVFTQLFAQAIMVYLPARELNMRPNMGLMWLSWWMAFIGLVVAALPL
190 200 210 220 230 240
Rma VTNKASVLYTSYAPLQAHWTYYVGLVFVVISTWLALLNMLLTWRGWKRENPGVRMPLLAH
Tth LANEATVLYTFYPPLKGHWAFYLGASVFVLSTWVSIYIVLDLWRRWKAANPGKVTPLVTY
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Tth MAVVFWLMWFLASLGLVLEAVLFLLPWSFGLVEGVDPLVARTLFWWTGHPIVYFWLLPAY
300 310 320 330 340 350 360
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Tth VAVPSLMTAFTVAASLEFAGRLRGGRGLFGWIRALPWDNPAFVAPVLGLLGFIPGGAGGI
420 430 440 450 460 470 480
Rma INASFTMNQVVHNTTWVPGHFHMTVGSAVAMTFMGVAYWMVPYLTGKKLWG--RKVALAS
Tth VNASFTLDYVVHNTAWVPGHFHLQVASLVTLTAMGSLYWLLPNLTGKPISDAQRRLGLAV

Figure 4A (cont.)

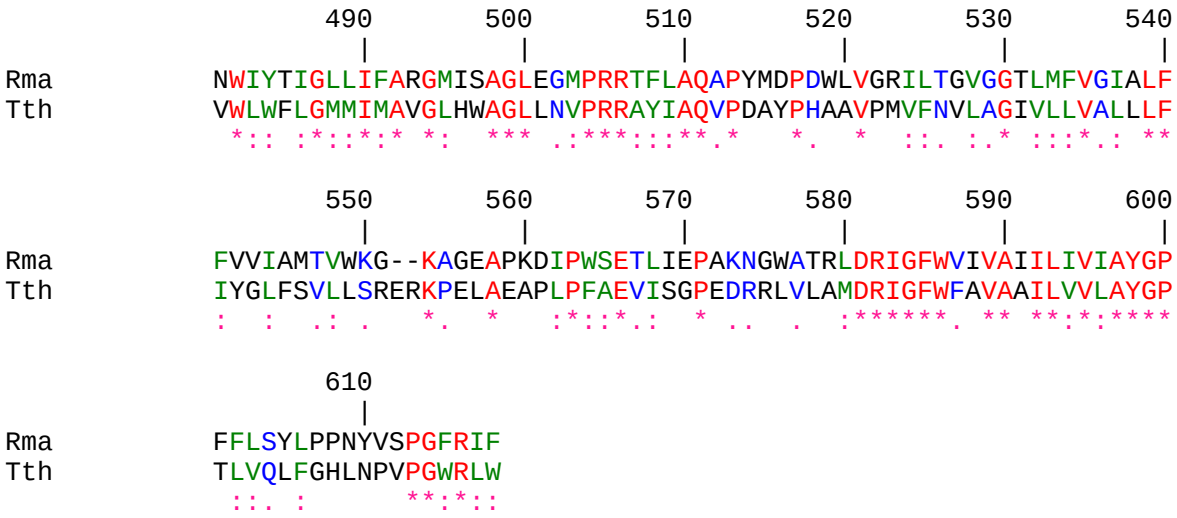


Figure 4B

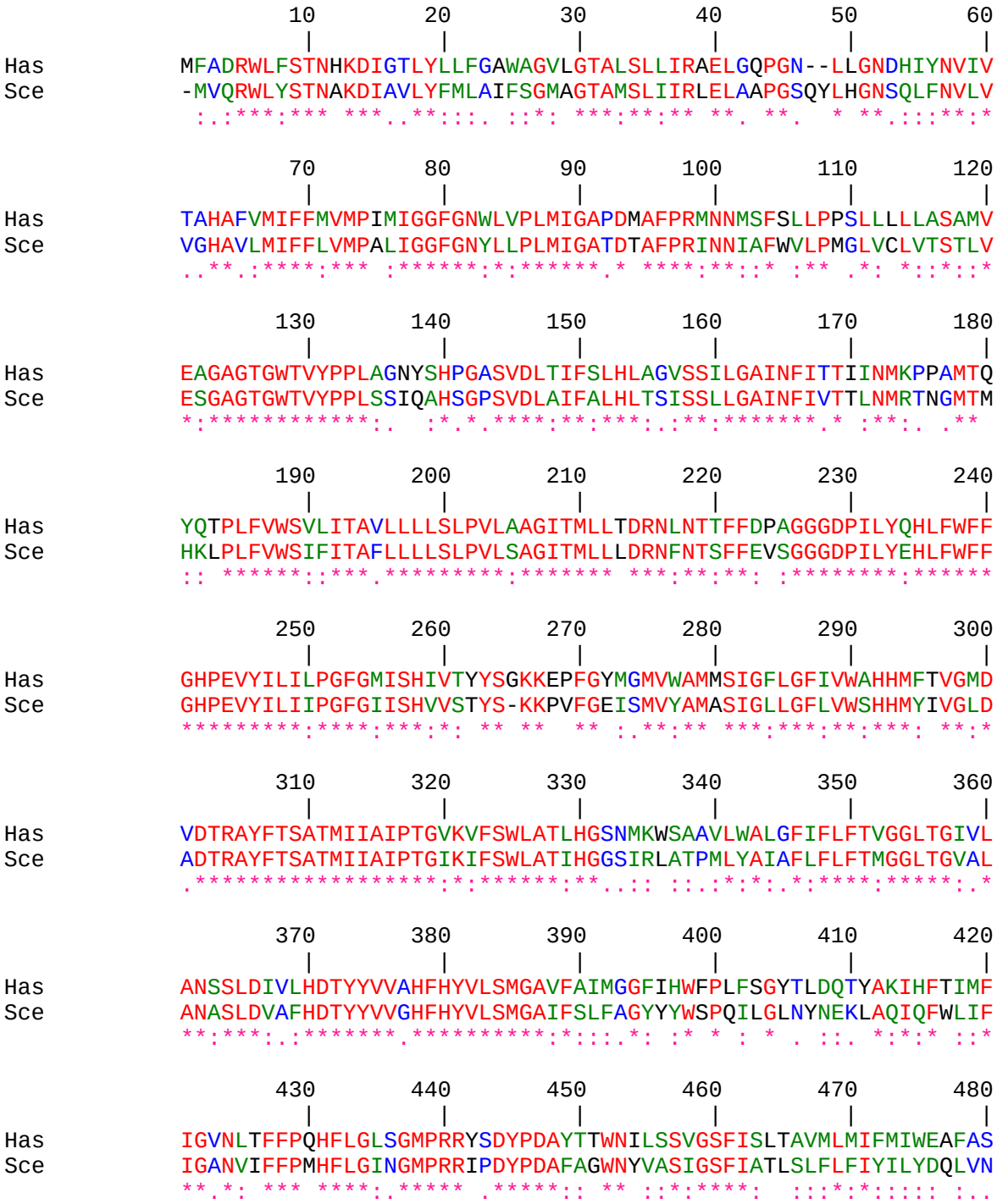

10 20 30 40 50 60
Has MFADRWLFSTNHKDIGTLYLLFGAWAGVLGTALSLLIRAELGQPGN--LLGNDHIYNVIV
Sce -MVQRWLYSTNAKDIAVLYFMLAIFSGMAGTAMSLIIRLELAAPGSQYLHGNSQLFNVLV
70 80 90 100 110 120
Has TAHAFVMIFFMVMPIMIGGFGNWLVPLMIGAPDMAFPRMNNMSFSLLPPSLLLLLASAMV
Sce VGHAVLMIFFLVMPALIGGFGNYLLPLMIGATDTAFPRINNIAFWVLPMGLVCLVTSTLV
130 140 150 160 170 180
Has EAGAGTGWTVYPPLAGNYSHPGASVDLTIFSLHLAGVSSILGAINFITTIINMKPPAMTQ
Sce ESGAGTGWTVYPPLSSIQAHSGPSVDLAIFALHLTSISSLLGAINFIVTTLNMRTNGMTM
190 200 210 220 230 240
Has YQTPLFVWSVLITAVLLLLSLPVLAAGITMLLTDRNLNTTFFDPAGGGDPILYQHLFWFF
Sce HKLPLFVWSIFITAFLLLLSLPVLSAGITMLLLDRNFNTSFFEVSGGGDPILYEHLFWFF
250 260 270 280 290 300
Has GHPEVYILILPGFGMISHIVTYYSGKKEPFGYMGMVWAMMSIGFLGFIVWAHHMFTVGMD
Sce GHPEVYILIIPGFGIISHVVSTYS-KKPVFGEISMVYAMASIGLLGFLVWSHHMYIVGLD
310 320 330 340 350 360
Has VDTRAYFTSATMIIAIPTGVKVFSWLATLHGSNMKWSAAVLWALGFIFLFTVGGLTGIVL
Sce ADTRAYFTSATMIIAIPTGIKIFSWLATIHGGSIRLATPMLYAIAFLFLFTMGGLTGVAL
370 380 390 400 410 420
Has ANSSLDIVLHDTYYVVAHFHYVLSMGAVFAIMGGFIHWFPLFSGYTLDQTYAKIHFTIMF
Sce ANASLDVAFHDTYYVVGHFHYVLSMGAIFSLFAGYYYWSPQILGLNYNEKLAQIQFWLIF
430 440 450 460 470 480
Has IGVNLTFFPQHFLGLSGMPRRYSDYPDAYTTWNILSSVGSFISLTAVMLMIFMIWEAFAS
Sce IGANVIFFPMHFLGINGMPRRIPDYPDAFAGWNYVASIGSFIATLSLFLFIYILYDQLVN

Figure 4B (cont.)

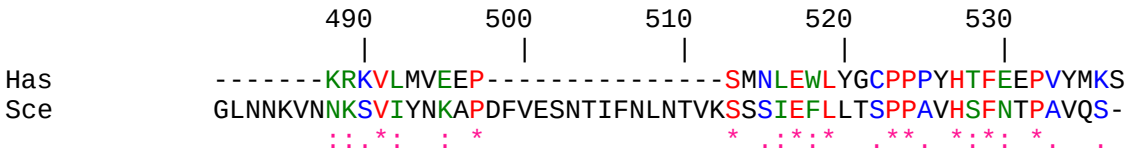


Figure 4C

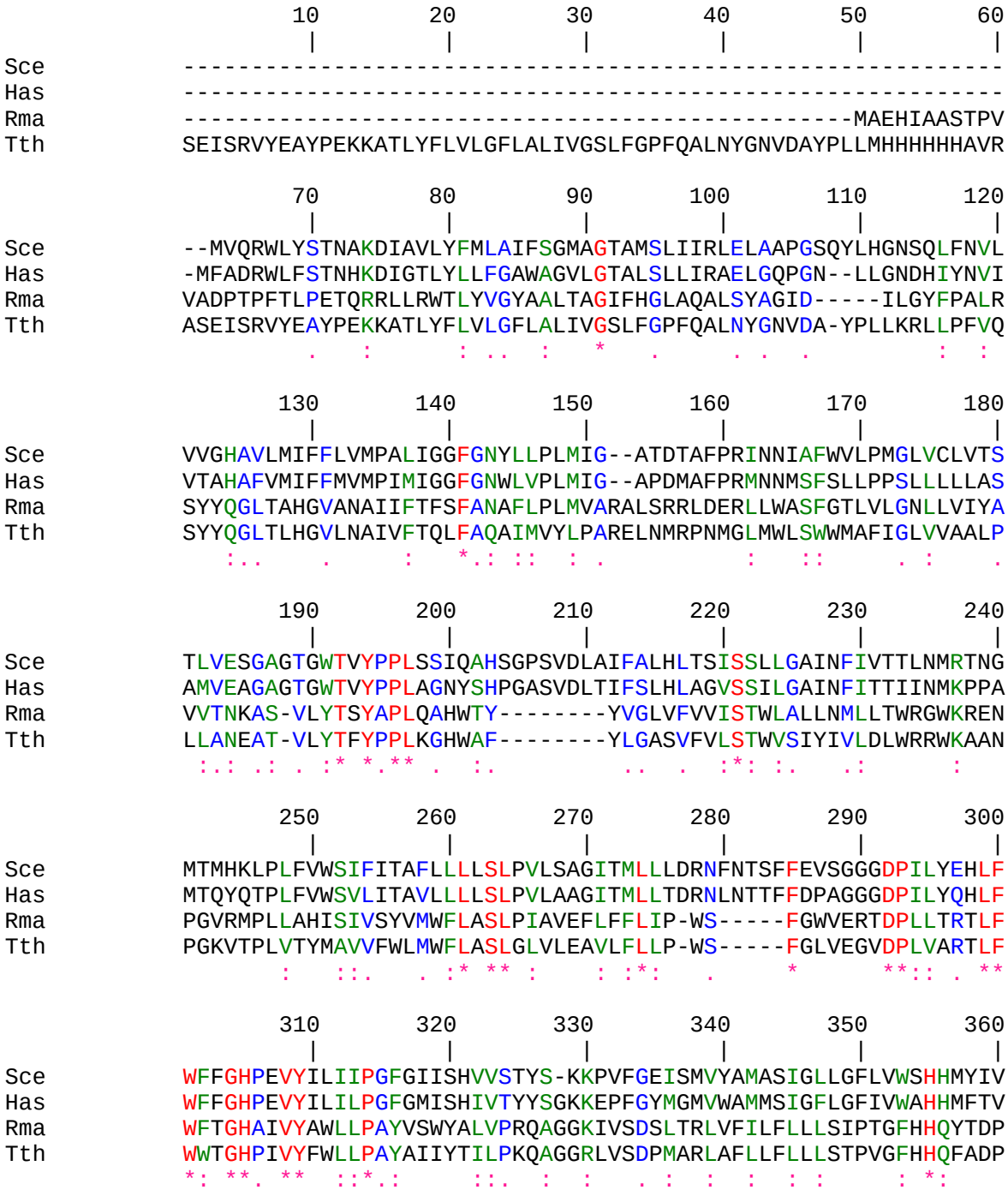

10 20 30 40 50 60
Sce ----------
Has ----------
Rma ----------MAEHIAASTPV
Tth SEISRVYEAYPEKKATLYFLVLGFLALIVGSLFGPFQALNYGNVDAYPLLMHHHHHHAVR
70 80 90 100 110 120
Sce --MVQRWLYSTNAKDIAVLYFMLAIFSGMAGTAMSLIIRLELAAPGSQYLHGNSQLFNVL
Has -MFADRWLFSTNHKDIGTLYLLFGAWAGVLGTALSLLIRAELGQPGN--LLGNDHIYNVI
Rma VADPTPFTLPETQRRLLRWTLYVGYAALTAGIFHGLAQALSYAGID-----ILGYFPALR
Tth ASEISRVYEAYPEKKATLYFLVLGFLALIVGSLFGPFQALNYGNVDA-YPLLKRLLPFVQ
130 140 150 160 170 180
Sce VVGHAVLMIFFLVMPALIGGFGNYLLPLMIG--ATDTAFPRINNIAFWVLPMGLVCLVTS
Has VTAHAFVMIFFMVMPIMIGGFGNWLVPLMIG--APDMAFPRMNNMSFSLLPPSLLLLLAS
Rma SYYQGLTAHGVANAIIFTFSFANAFLPLMVARALSRRLDERLLWASFGTLVLGNLLVIYA
Tth SYYQGLTLHGVLNAIVFTQLFAQAIMVYLPARELNMRPNMGLMWLSWWMAFIGLVVAALP
190 200 210 220 230 240
Sce TLVESGAGTGWTVYPPLSSIQAHSGPSVDLAIFALHLTSISSLLGAINFIVTTLNMRTNG
Has AMVEAGAGTGWTVYPPLAGNYSHPGASVDLTIFSLHLAGVSSILGAINFITTIINMKPPA
Rma VVTNKAS-VLYTSYAPLQAHWTY--------YVGLVFVVISTWLALLNMLLTWRGWKREN
Tth LLANEAT-VLYTFYPPLKGHWAF--------YLGASVFVLSTWVSIYIVLDLWRRWKAAN
250 260 270 280 290 300
Sce MTMHKLPLFVWSIFITAFLLLLSLPVLSAGITMLLLDRNFNTSFFEVSGGGDPILYEHLF
Has MTQYQTPLFVWSVLITAVLLLLSLPVLAAGITMLLTDRNLNTTFFDPAGGGDPILYQHLF
Rma PGVRMPLLAHISIVSYVMWFLASLPIAVEFLFFLIP-WS-----FGWVERTDPLLTRTLF
Tth PGKVTPLVTYMAVVFWLMWFLASLGLVLEAVLFLLP-WS-----FGLVEGVDPLVARTLF
310 320 330 340 350 360
Sce WFFGHPEVYILIIPGFGIISHVVSTYS-KKPVFGEISMVYAMASIGLLGFLVWSHHMYIV
Has WFFGHPEVYILILPGFGMISHIVTYYSGKKEPFGYMGMVWAMMSIGFLGFIVWAHHMFTV
Rma WFTGHAIVYAWLLPAYVSWYALVPRQAGGKIVSDSLTRLVFILFLLLSIPTGFHHQYTDP
Tth WWTGHPIVYFWLLPAYAIIYTILPKQAGGRLVSDPMARLAFLLFLLLSTPVGFHHQFADP

Figure 4C (cont.)

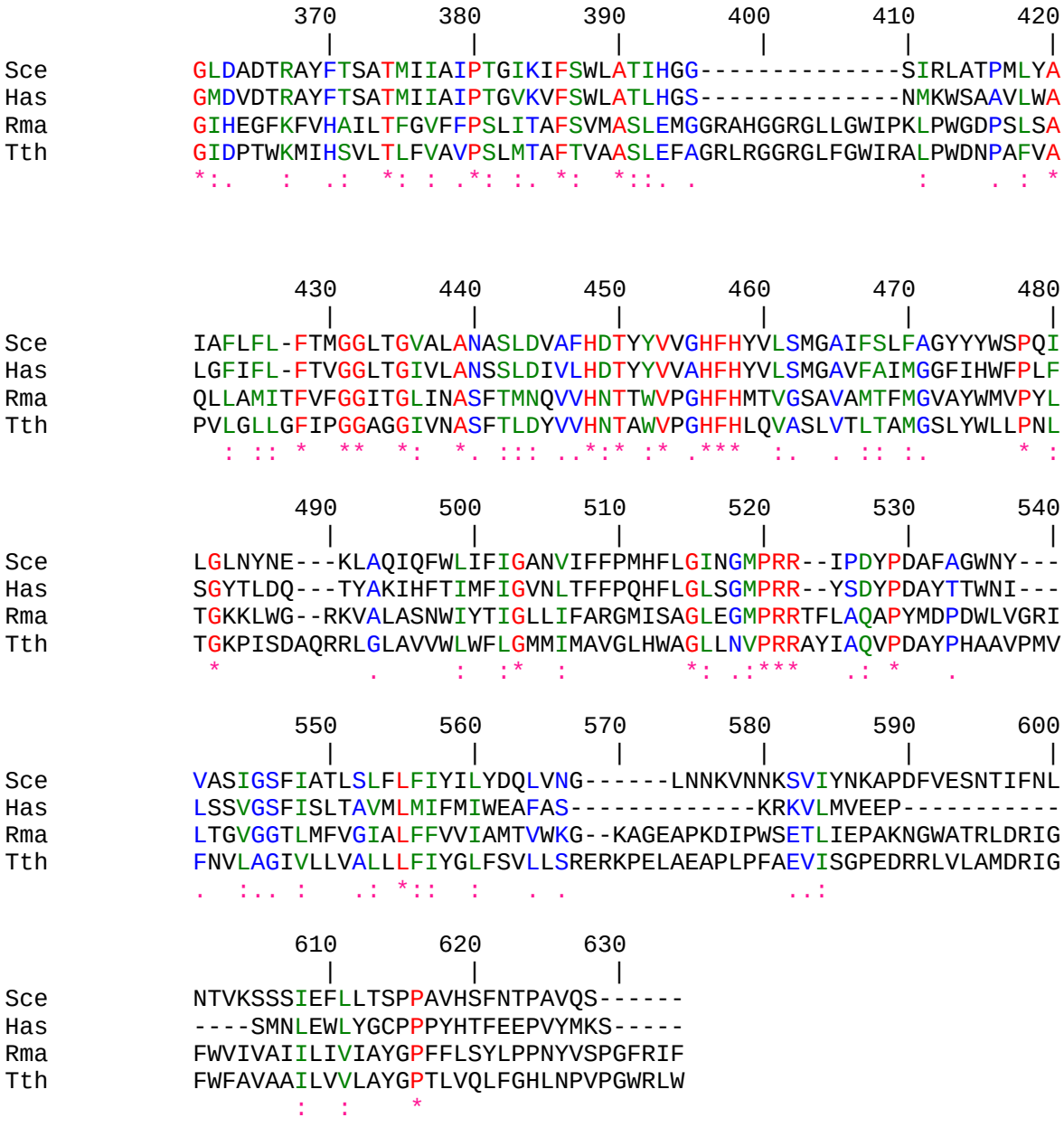


Figure 5

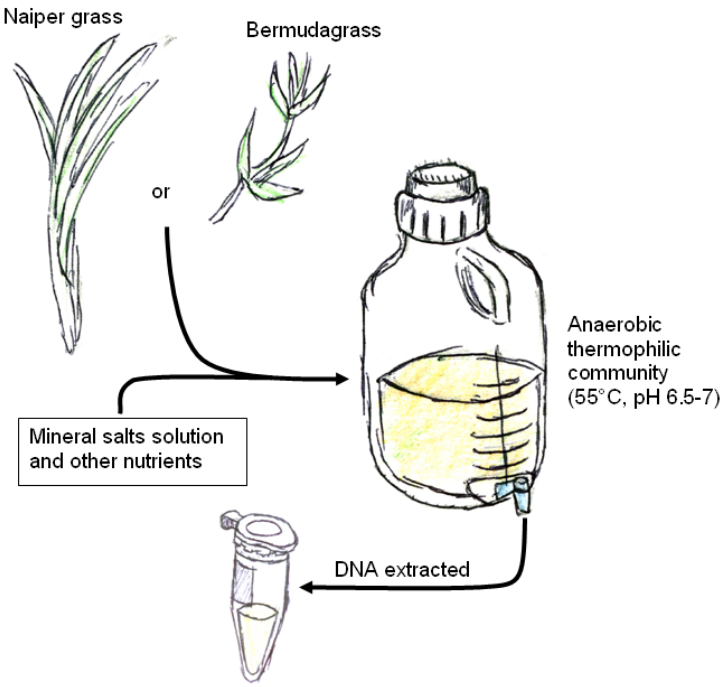
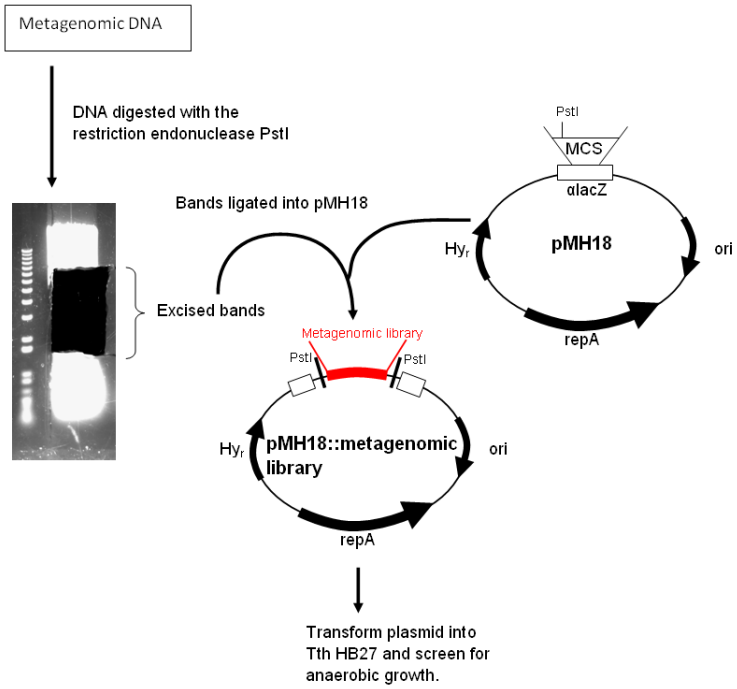

Naiper grass
Bermudagrass
or
Anaerobic
thermophilic
community
(55°C, pH 6.5-7)
Mineral salts solution
and other nutrients
DNA extracted

Figure 6

A


Metagenomic DNA
DNA digested with the restriction endonuclease PstI
Excised bands
Bands ligated into pMH18
PstI
MCS
αlacZ
Hyr
pMH18
ori
repA
Metagenomic library
PstI
PstI
Hyr
pMH18::metagenomic library
ori
repA
Transform plasmid into Tth HB27 and screen for anaerobic growth.

B

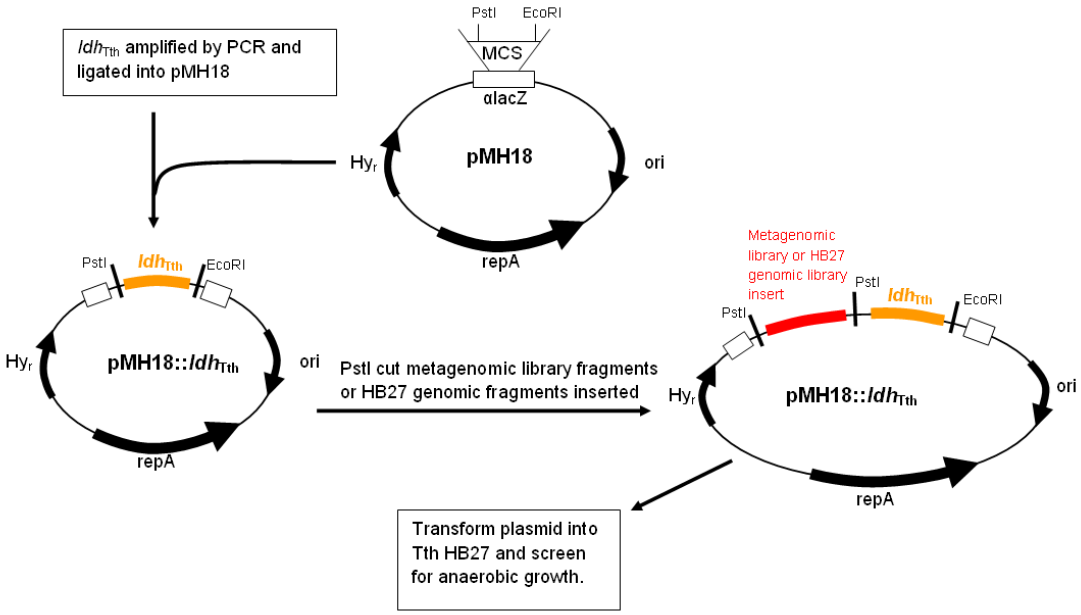

ldhTth amplified by PCR and ligated into pMH18
PstI
EcoRI
MCS
αlacZ
Hyr
pMH18
ori
repA
PstI
ldhTth
EcoRI
Hyr
pMH18::ldhTth
ori
repA
PstI cut metagenomic library fragments or HB27 genomic fragments inserted
Metagenomic library or HB27 genomic library insert
PstI
PstI
ldhTth
EcoRI
Hyr
pMH18::ldhTth
ori
repA
Transform plasmid into Tth HB27 and screen for anaerobic growth.

Figure 7

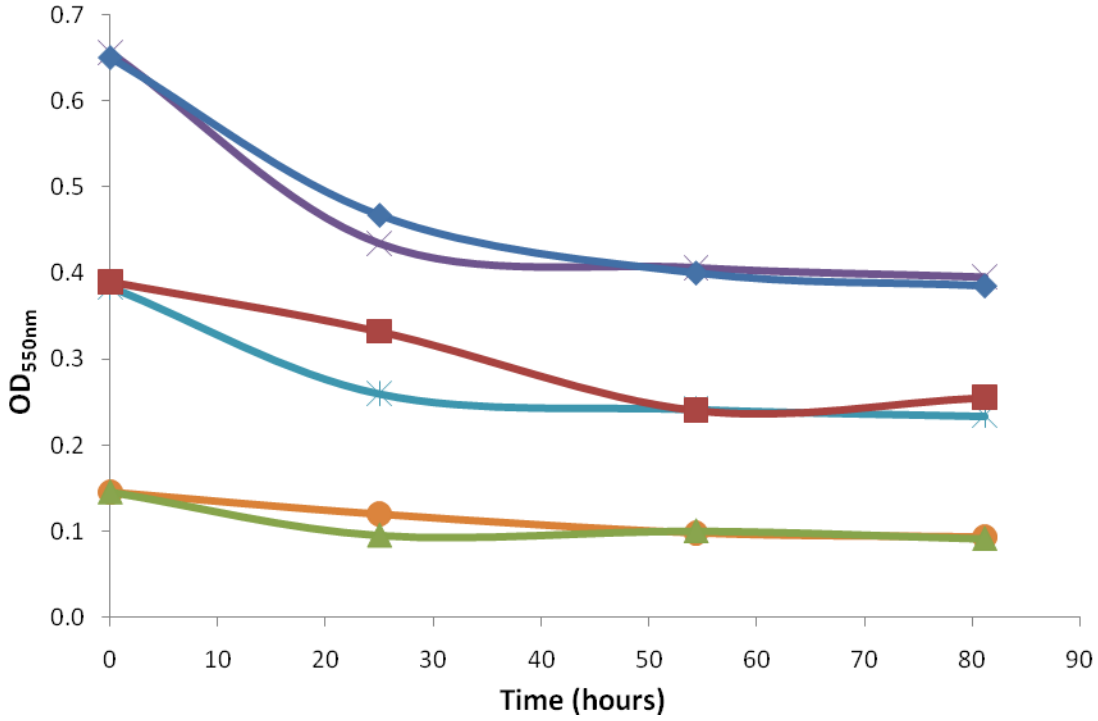
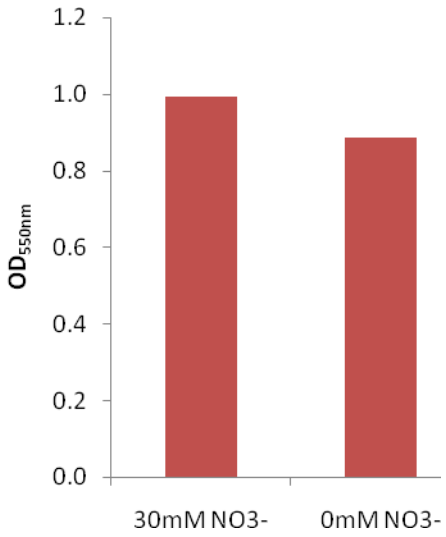

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD550nm
0
10
20
30
40
50
60
70
80
90
Time (hours)

Figure 8

A


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD550nm
30mM NO3-
0mM NO3-

B

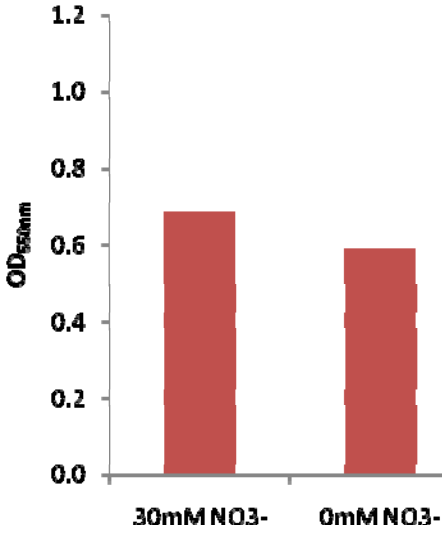

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD550nm
30mM NO3-
0mM NO3-

Figure 9

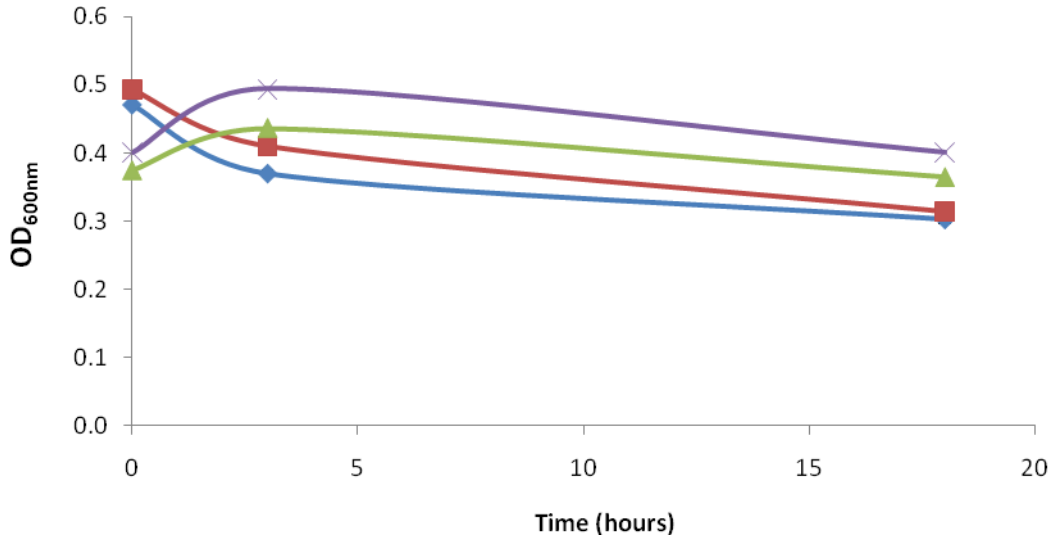


Figure 10

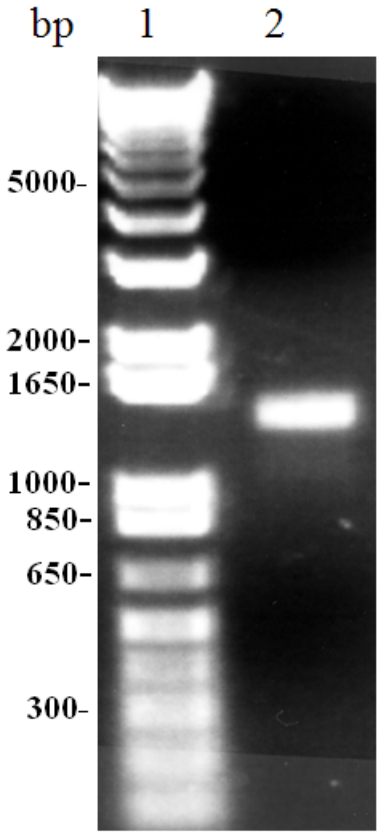


Figure 11

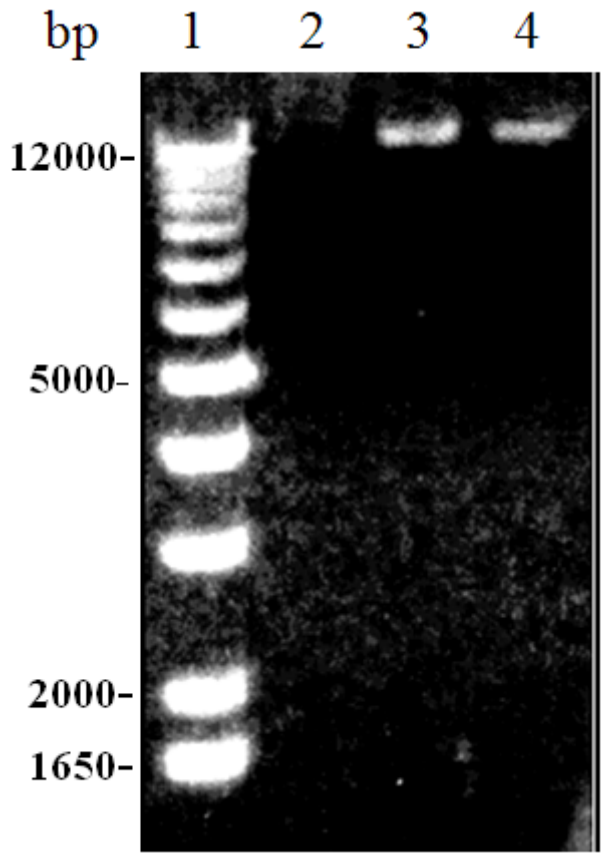


Figure 12

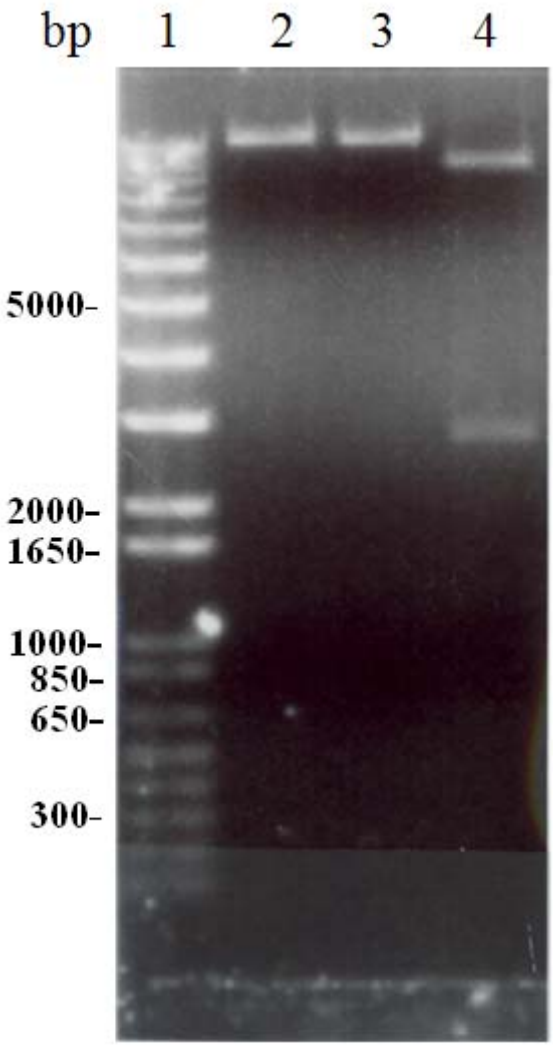


Figure 13

A **B**

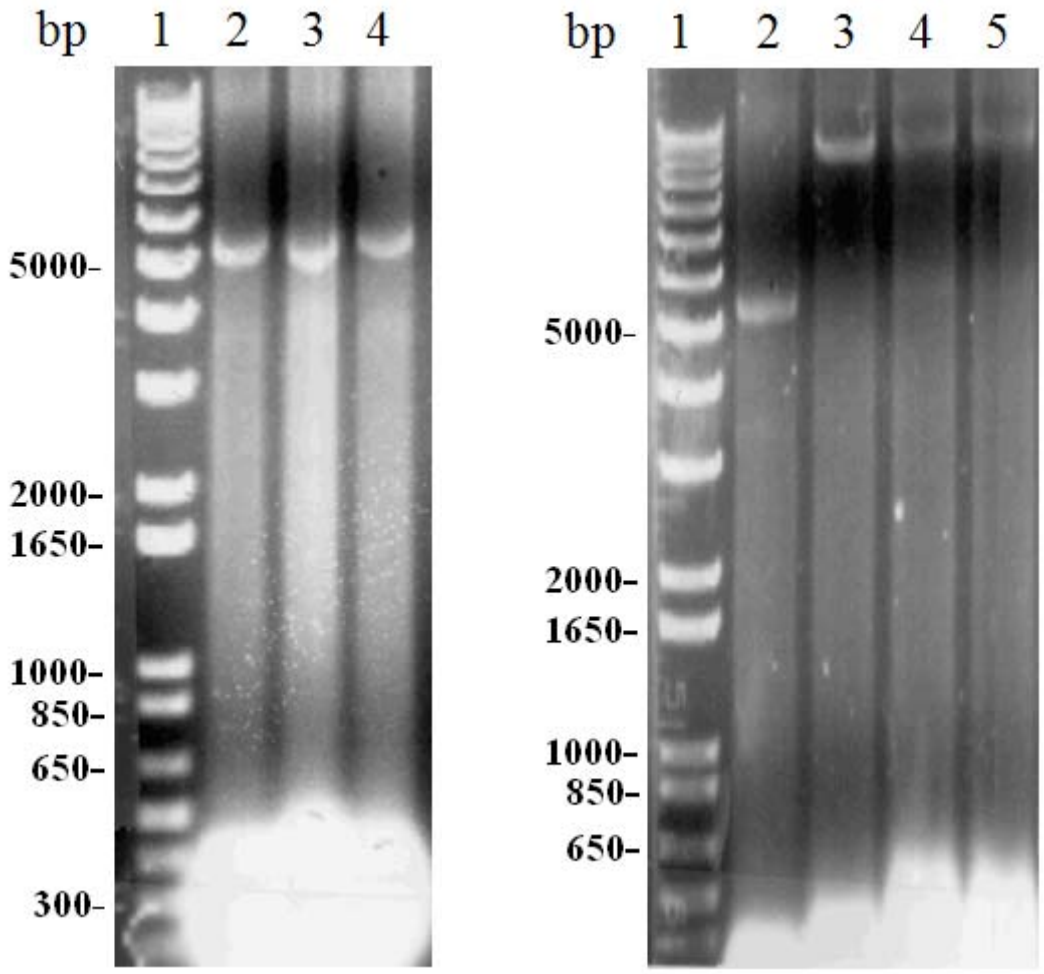

bp 1 2 3 4
5000-
2000-
1650-
1000-
850-
650-
300-
bp 1 2 3 4 5
5000-
2000-
1650-
1000-
850-
650-

Figure 14

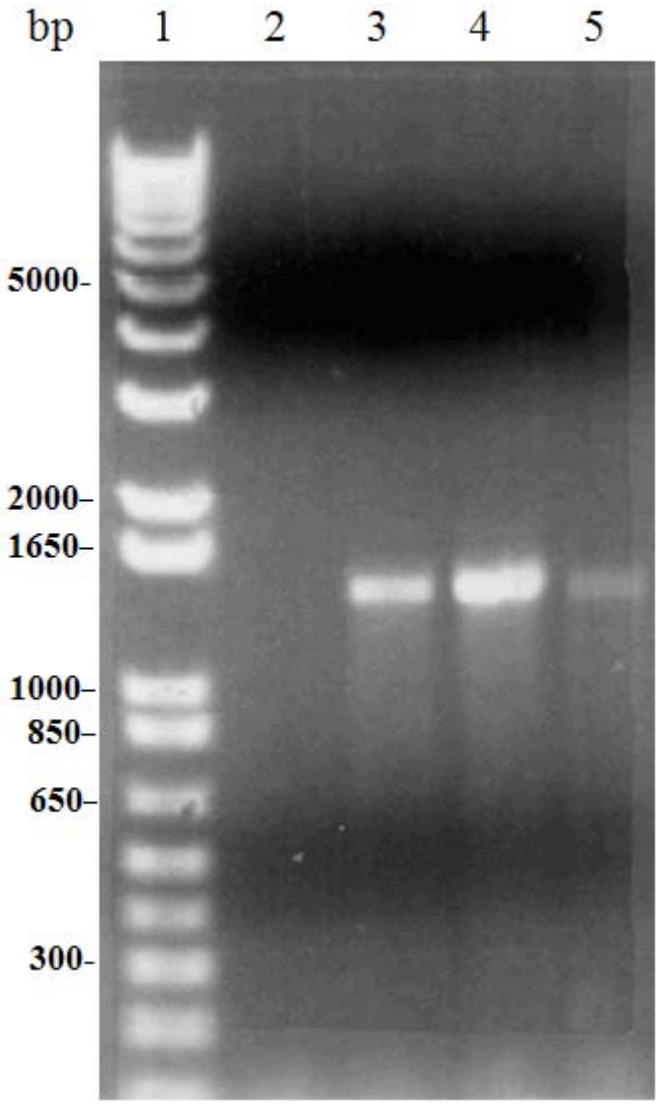


Figure 15

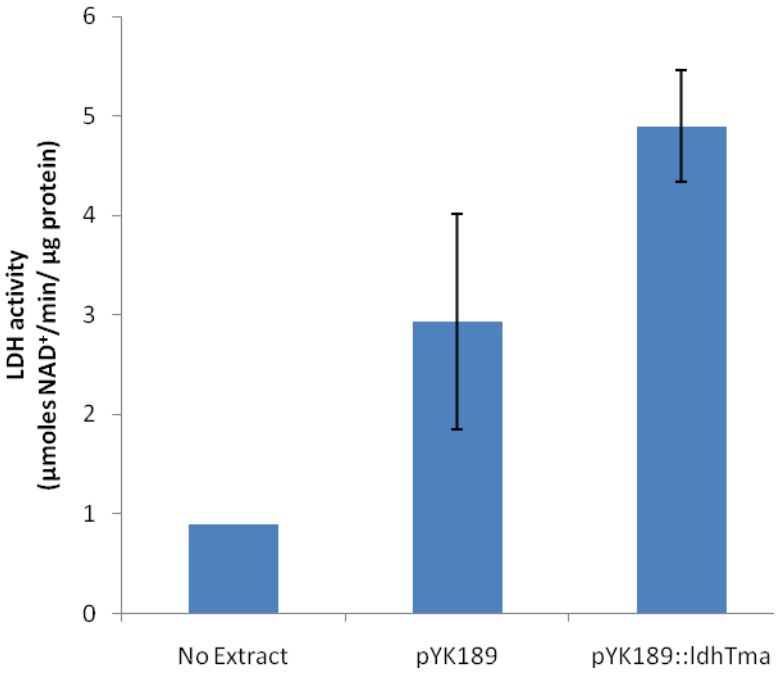
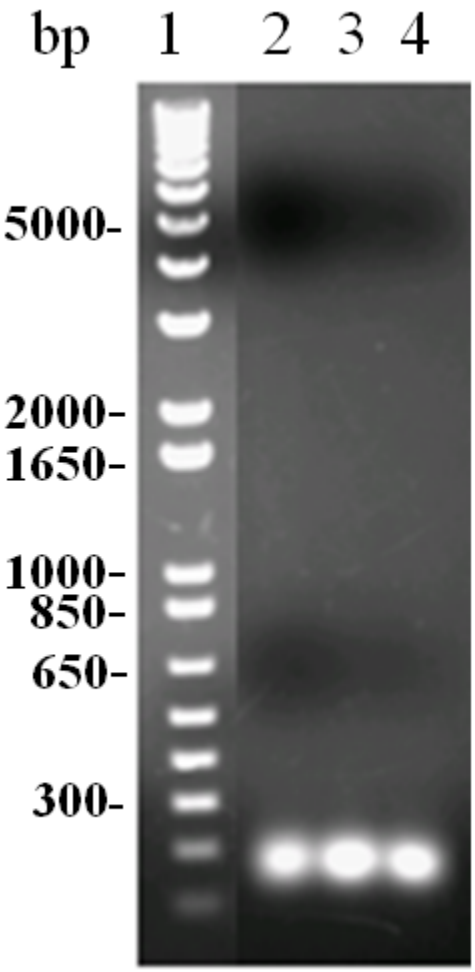

LDH activity
(µmoles NAD+/min/ µg protein)
6
5
4
3
2
1
0
No Extract
pYK189
pYK189::ldhTma

Figure 16

A


bp
1
2
3
4
5000-
2000-
1650-
1000-
850-
650-
300-

B

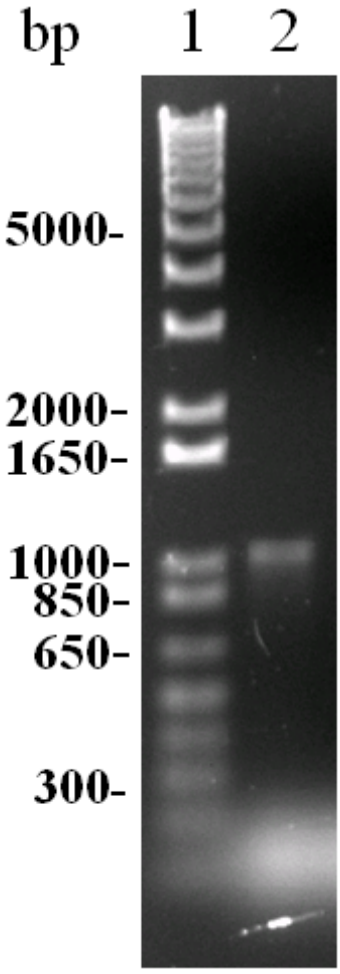

bp
1
2
5000-
2000-
1650-
1000-
850-
650-
300-

Figure 17

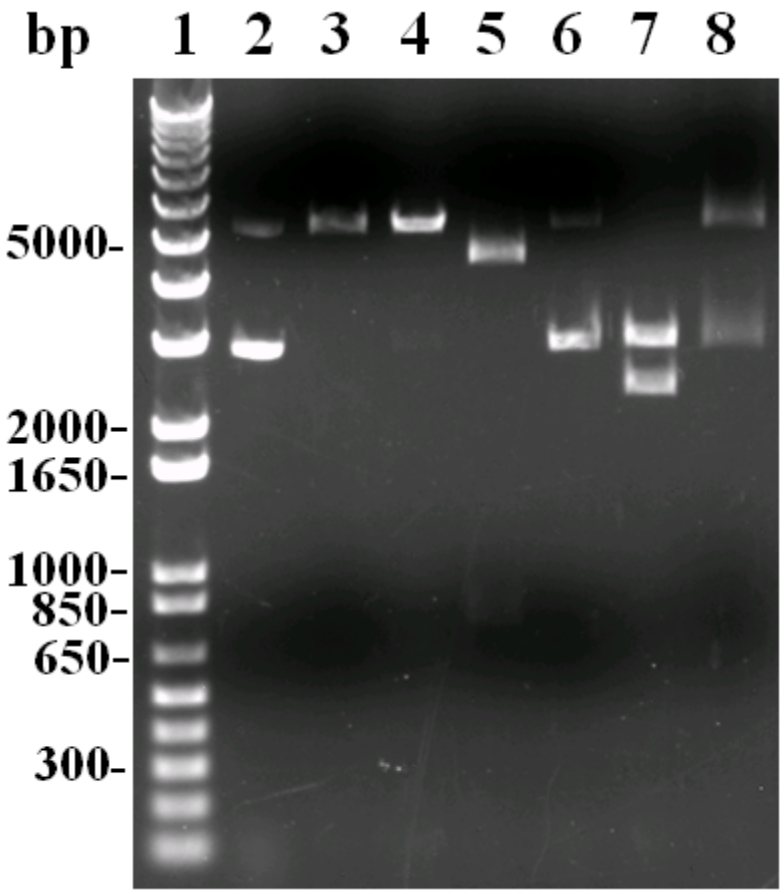


Figure 18

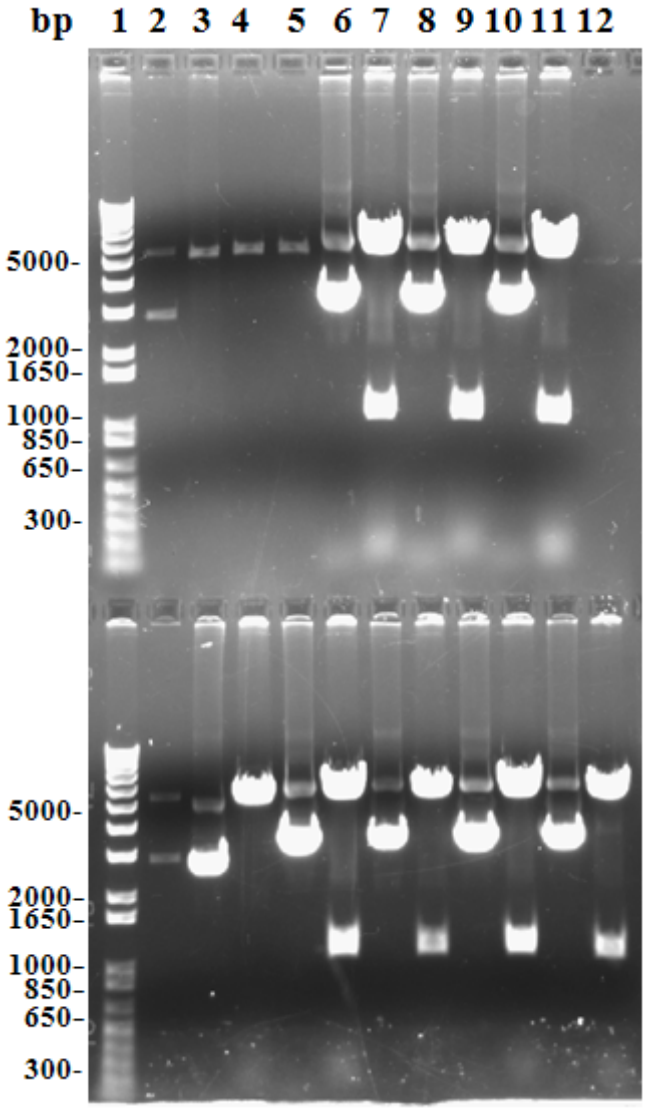

bp 1 2 3 4 5 6 7 8 9 10 11 12
5000-
2000-
1650-
1000-
850-
650-
300-
5000-
2000-
1650-
1000-
850-
650-
300-

Figure 19

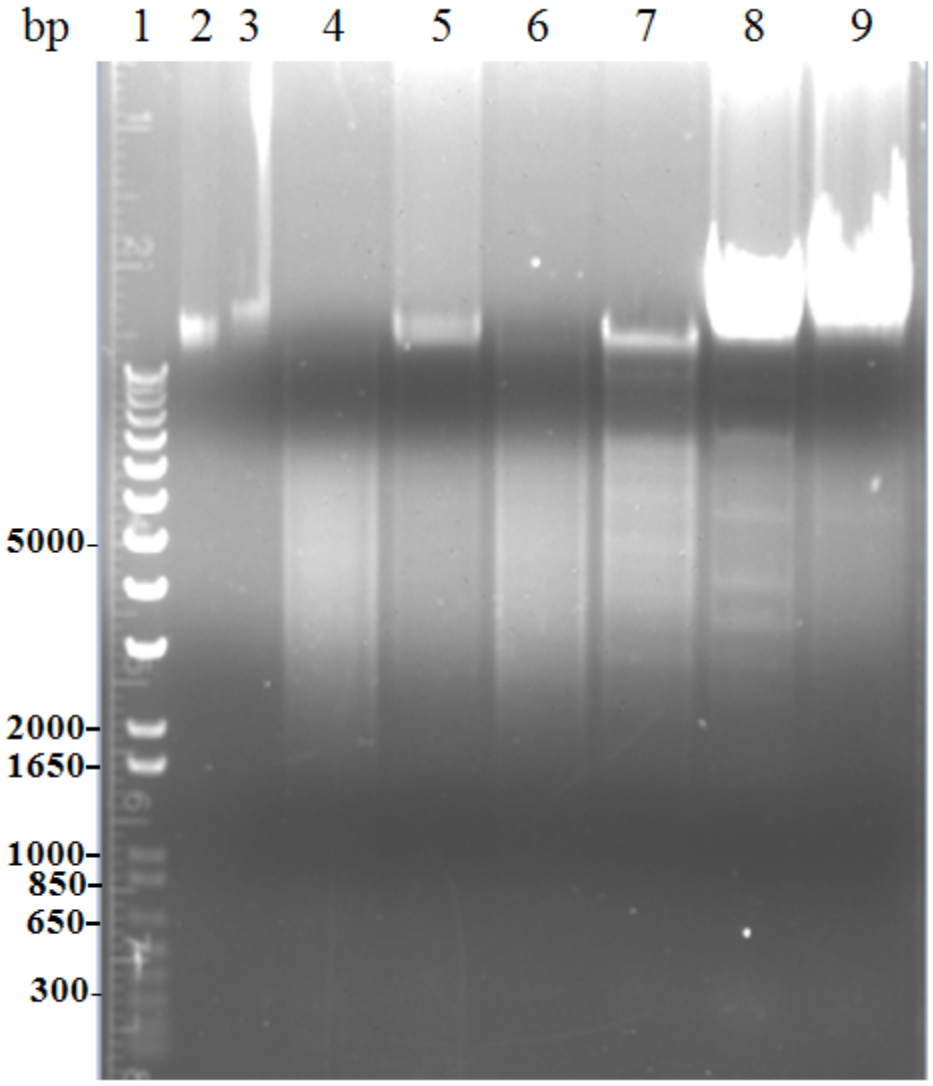


Figure 20

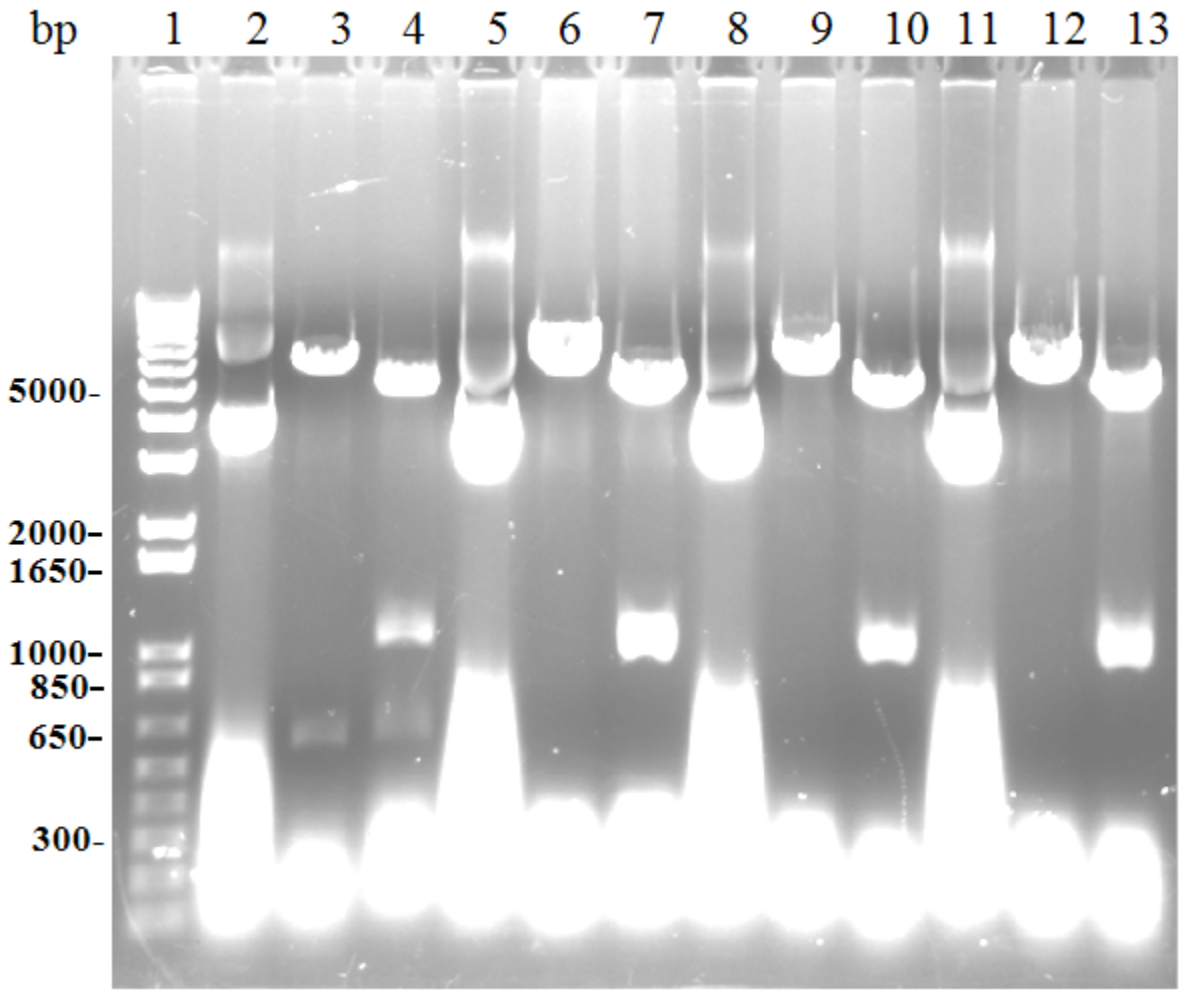


Figure 21

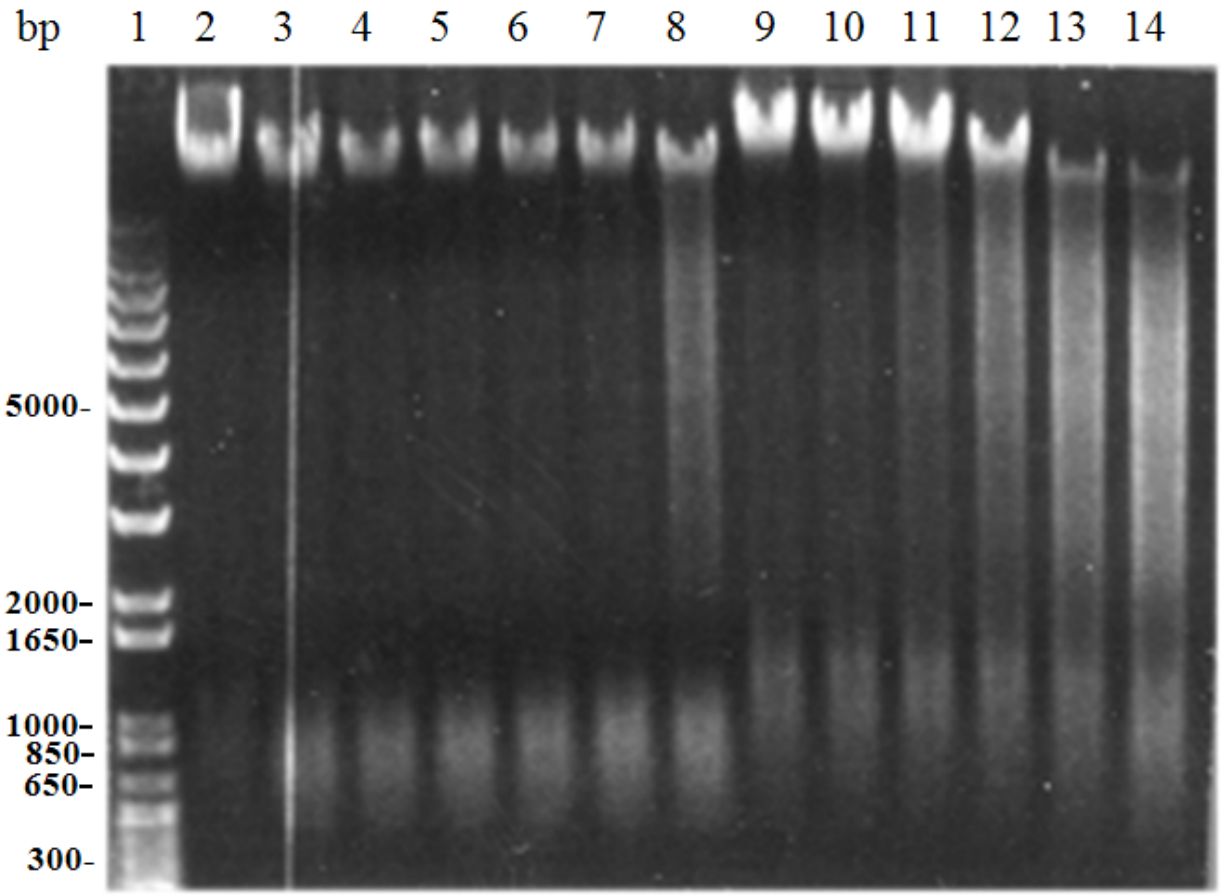
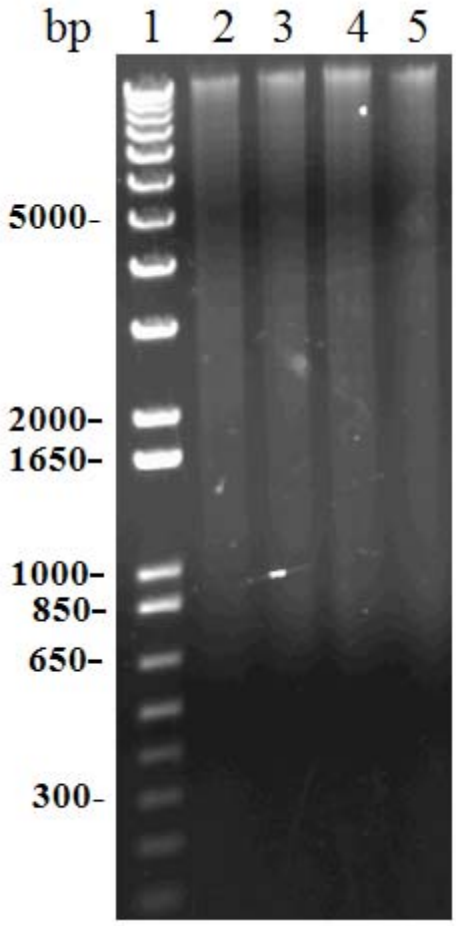


Figure 22

A


bp
1
2
3
4
5
5000-
2000-
1650-
1000-
850-
650-
300-

B

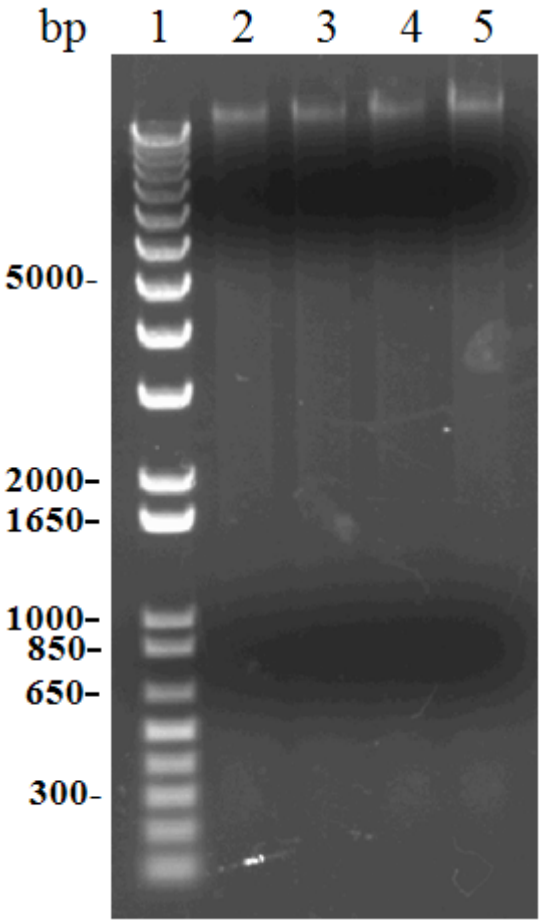

bp
1
2
3
4
5
5000-
2000-
1650-
1000-
850-
650-
300-

Figure 23

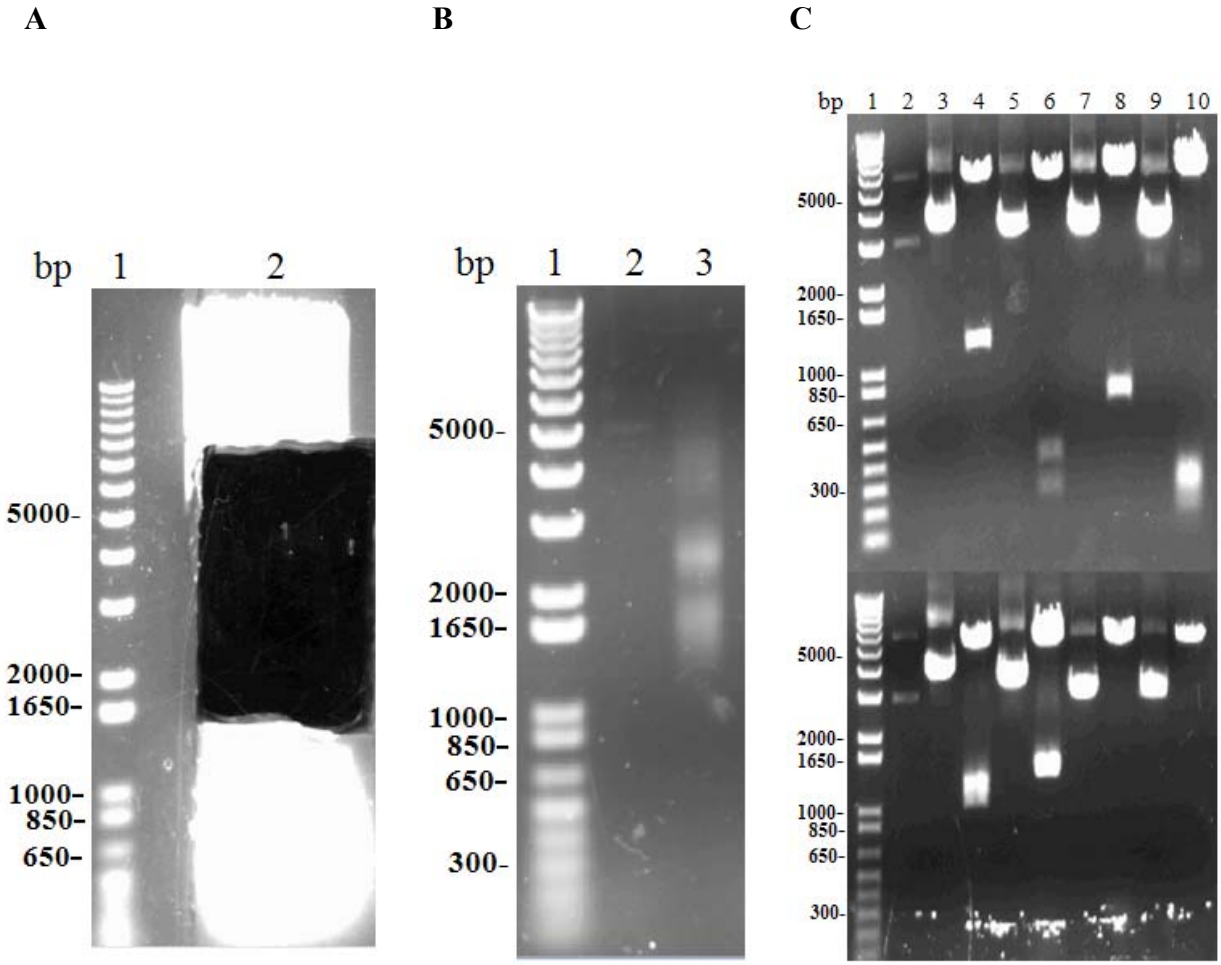

A
B
C
bp 1 2
5000
2000
1650
1000
850
650
bp 1 2 3
5000
2000
1650
1000
850
650
300
bp 1 2 3 4 5 6 7 8 9 10
5000
2000
1650
1000
850
650
300
5000
2000
1650
1000
850
650
300

Figure 24

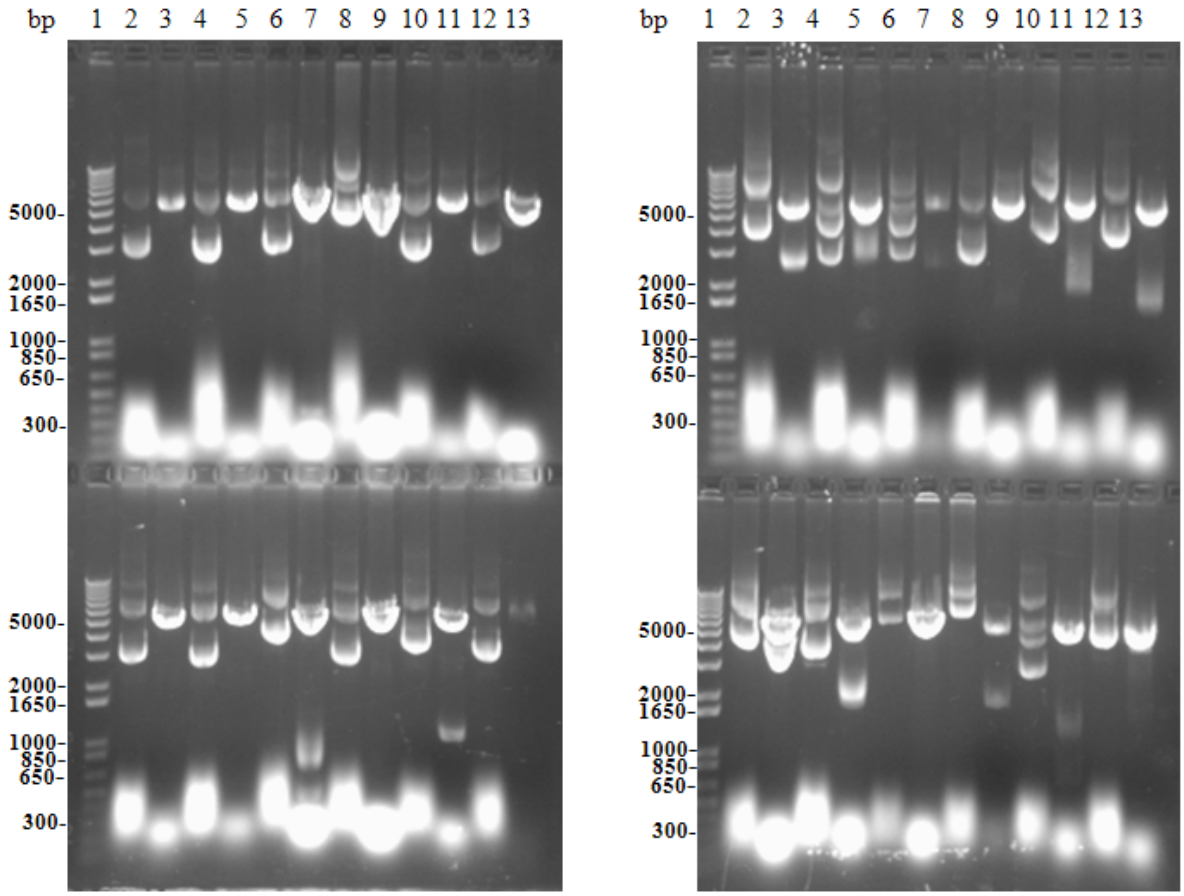

bp 1 2 3 4 5 6 7 8 9 10 11 12 13
5000
2000
1650
1000
850
650
300
bp 1 2 3 4 5 6 7 8 9 10 11 12 13
5000
2000
1650
1000
850
650
300

Figure 25

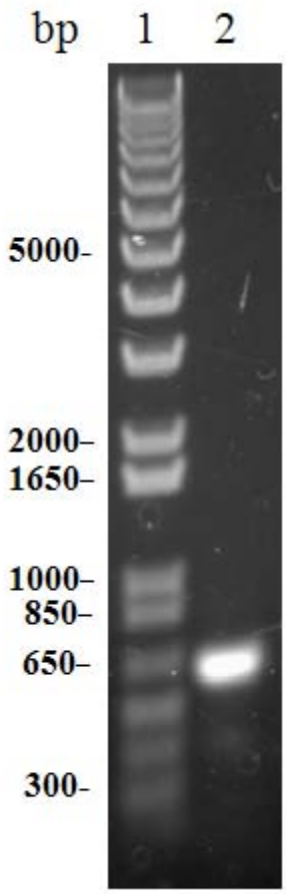


Figure 26

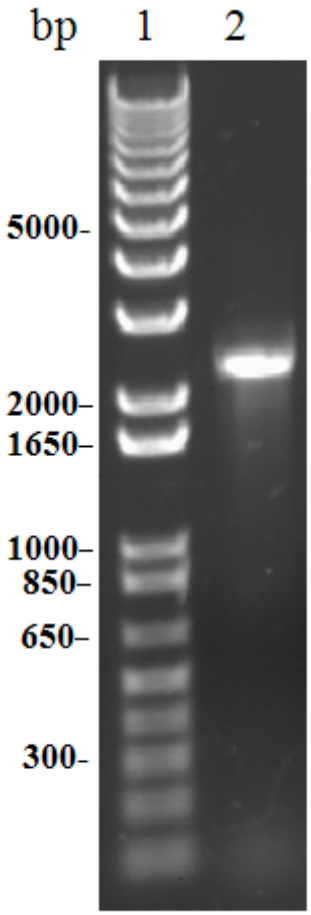
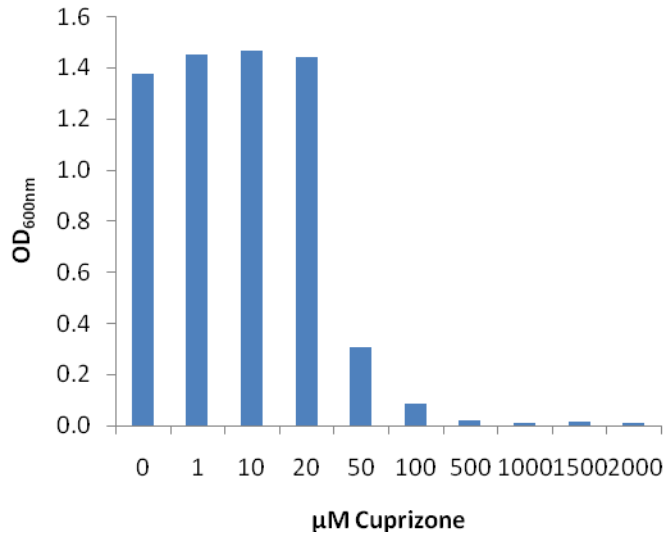


Figure 27

A


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm
0 1 10 20 50 100 500 1000 1500 2000
µM Cuprizone

B

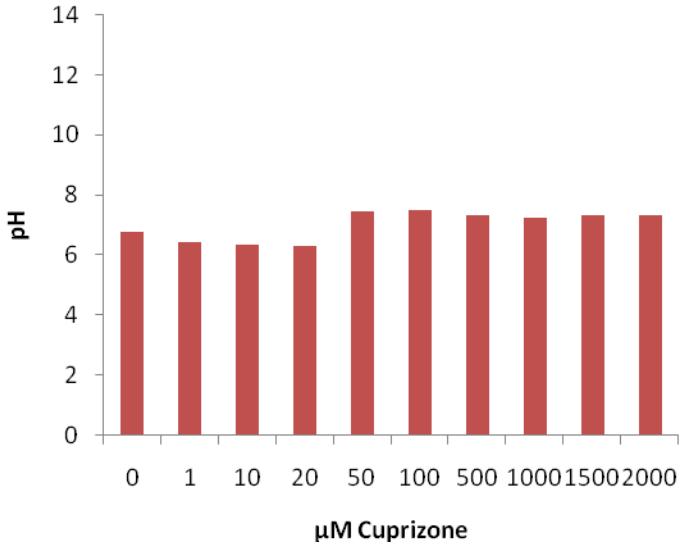

14
12
10
8
6
4
2
0
pH
0 1 10 20 50 100 500 1000 1500 2000
µM Cuprizone

Figure 28

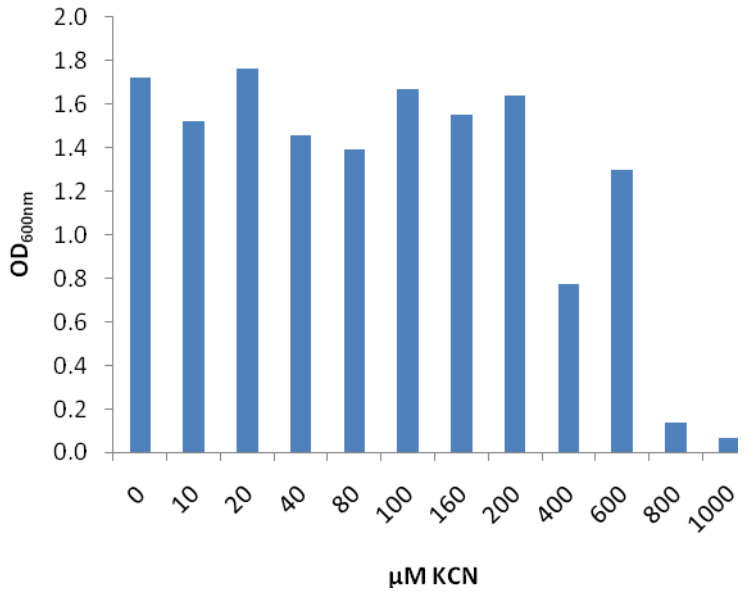
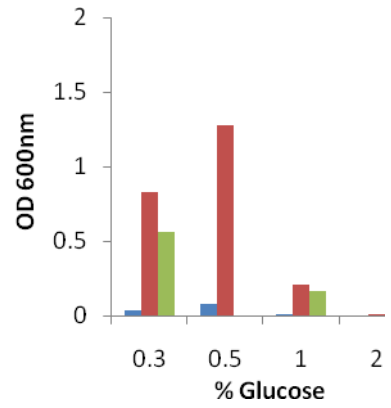
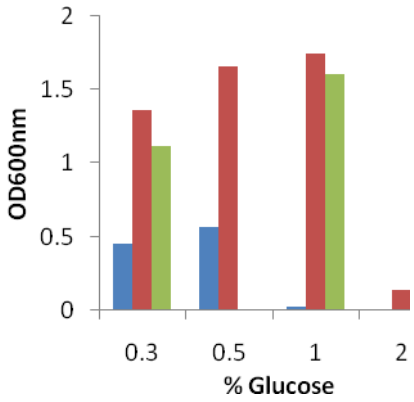

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm
0
10
20
40
80
100
160
200
400
600
800
1000
µM KCN

Figure 29

A



B



C

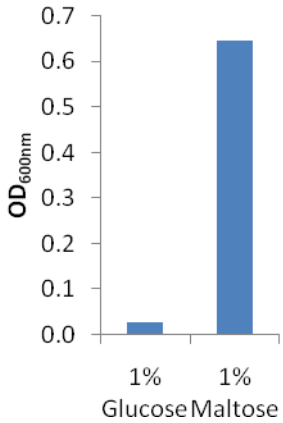
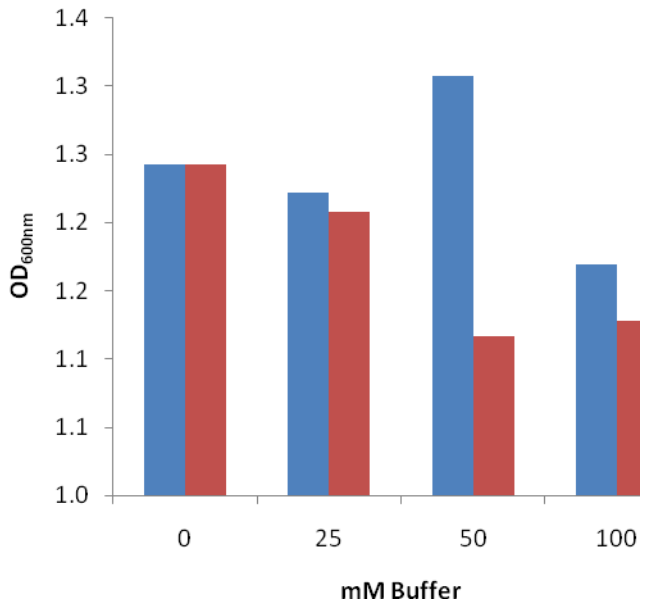


Figure 30

A


1.4
1.3
1.3
1.2
1.2
1.1
1.1
1.0
OD600nm
0
25
50
100
mM Buffer

B

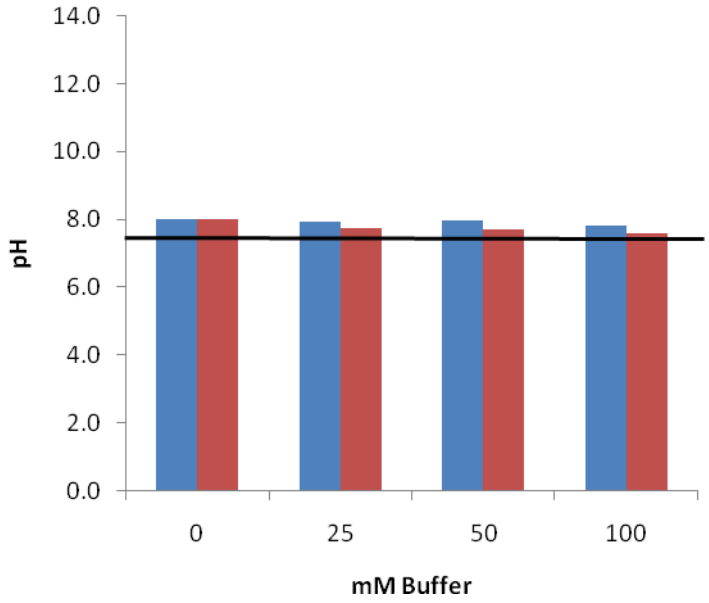

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
pH
0
25
50
100
mM Buffer

Figure 31

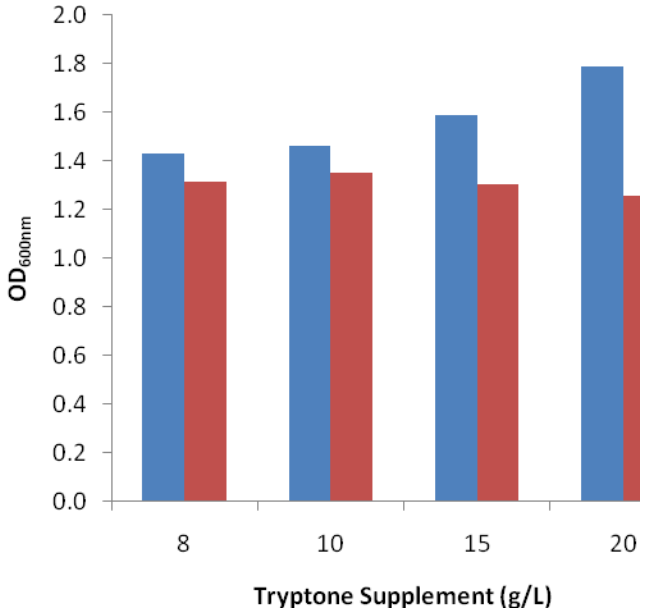

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm
8
10
15
20
Tryptone Supplement (g/L)

Figure 32

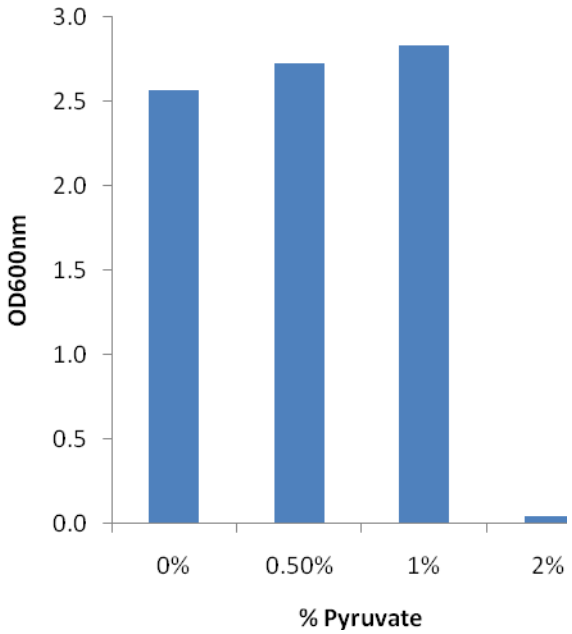
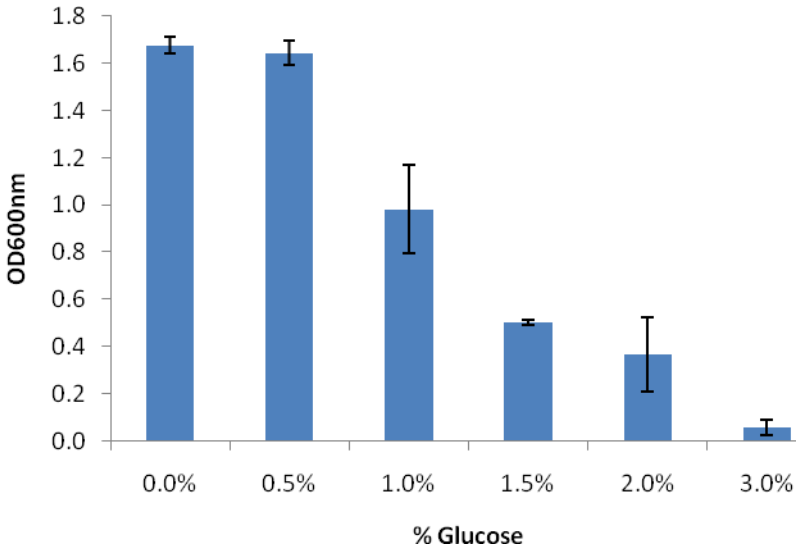


Figure 33

A


OD600nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0%
0.5%
1.0%
1.5%
2.0%
3.0%
% Glucose

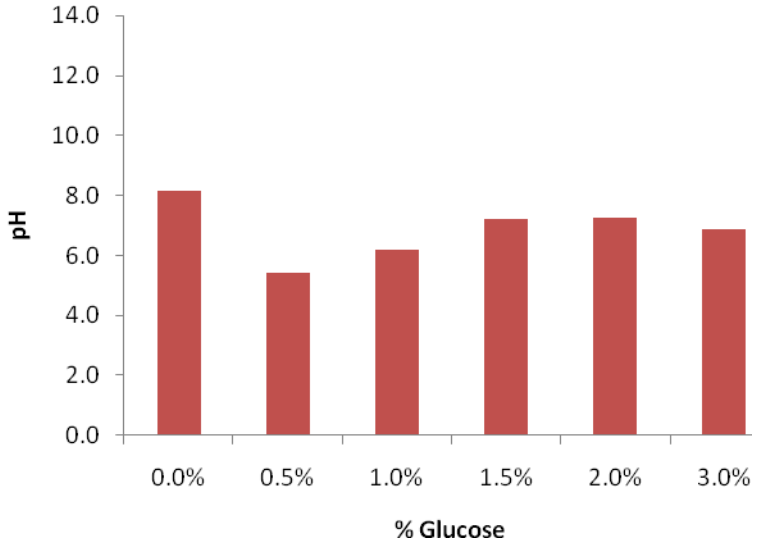

pH
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0.0%
0.5%
1.0%
1.5%
2.0%
3.0%
% Glucose

Figure 34

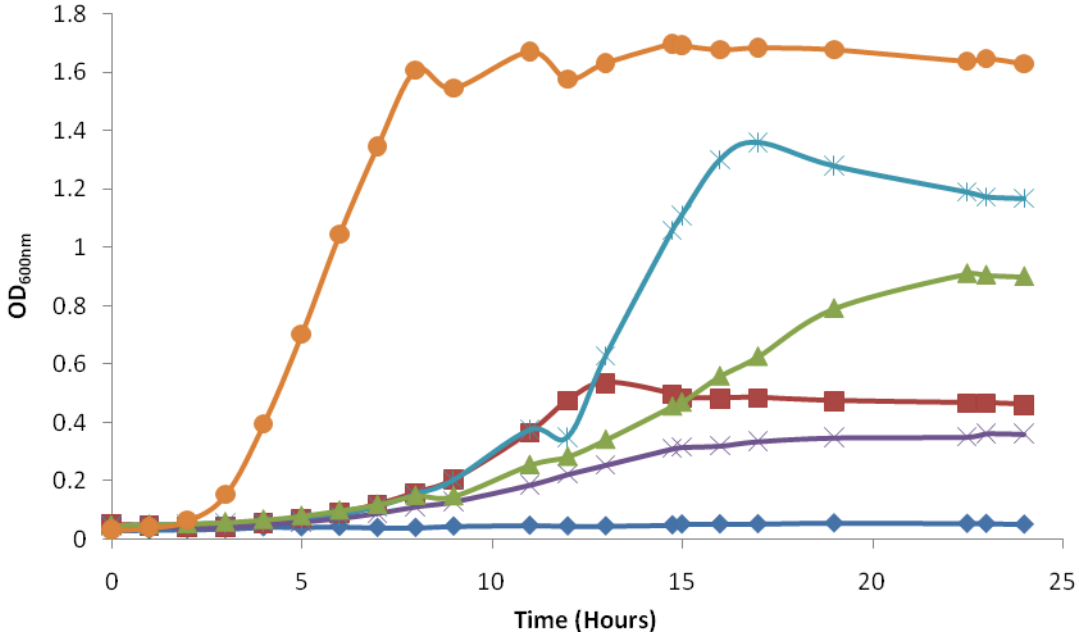
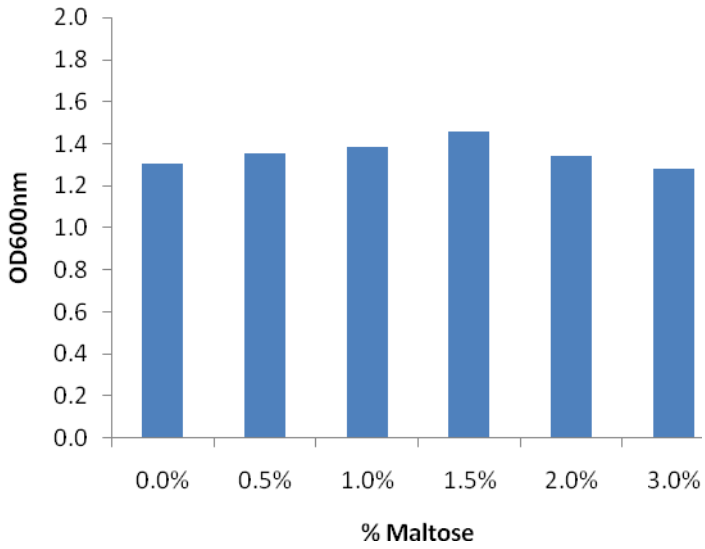


Figure 35

A


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm
0.0%
0.5%
1.0%
1.5%
2.0%
3.0%
% Maltose

B

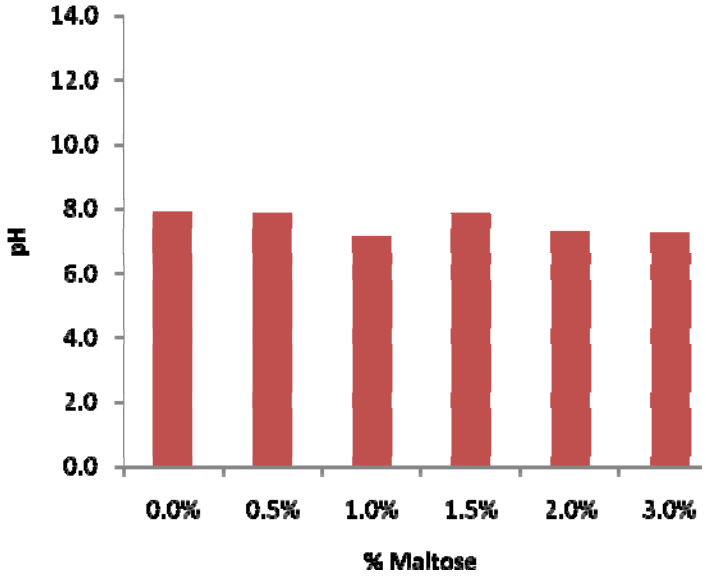
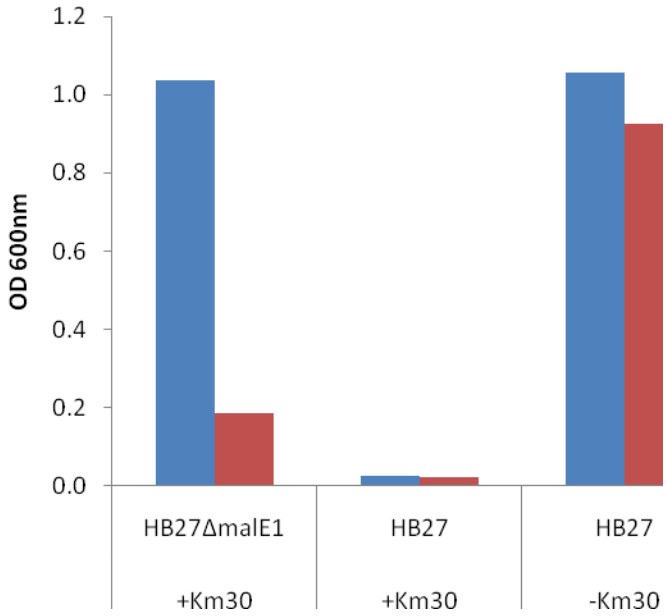

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
pH
0.0%
0.5%
1.0%
1.5%
2.0%
3.0%
% Maltose

Figure 37


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 600nm
HB27ΔmalE1
+Km30
HB27
+Km30
HB27
-Km30