Jan 31, 2019 rmk

Extreme Thermophiles as Emerging Metabolic Engineering Platforms

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Submitted to: ***Curr Opin Biotechnol - Issue on Tissue, Cell and Pathway Engineering 2019***

Keywords: Extreme thermophiles, metabolic engineering, industrial biotechnology

COBIOT_2018_146

Submitted: October, 2018

Revision submitted: February, 2019

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Abstract

Going forward, industrial biotechnology must consider non-model metabolic engineering platforms if it is to have maximal impact. This will include microorganisms that natively possess strategic physiological and metabolic features but lack either molecular genetic tools or such tools are rudimentary, requiring further development. If non-model platforms are successfully deployed, new avenues for production of fuels and chemicals from renewable feedstocks or waste materials will emerge. Here, the challenges and opportunities for extreme thermophiles as metabolic engineering platforms are discussed.

Introduction

Microbial metabolic engineering has achieved notable scientific successes (e.g., [1,2]), but is still limited by the challenges of bringing laboratory successes to industrial scale. Fortunately, genome-scale modeling, ever-improving -omics tools, and increased understanding of microbial metabolism can be leveraged to push past barriers and allow metabolic engineering to reach its potential. Such advances have opened the door for utilization of non-model metabolic engineering platforms, including extreme thermophiles [3]. Here, we focus on metabolic engineering opportunities for microorganisms growing optimally above 70°C, primarily from the genera *Caldicellulosiruptor, Pyrococcus, Thermococcus, Thermotoga,* and *Sulfolobus*.

Genetic systems of extremely thermophilic organisms

Utilization of extremely thermophilic microbes as metabolic engineering platforms requires establishing genetic techniques for generating recombinant strains (**Table 1**). First, this requires that the genome sequence of the selected organism be available to guide targeted mutations. Next, the microbe must be culturable at high temperatures on solid media at a sufficient efficiency to select successful clones. As many extreme thermophiles can take several

days to generate colonies, this may become a rate-limiting step. Once a satisfactory plating efficiency is reached, the transformation method must be chosen, and its efficiency evaluated. While there are some naturally competent extreme thermophiles, such as *Pyrococcus furiosus* COM1 [4], *T. kodakarensis* [5], and *Thermotoga sp. RQ7* [6], electroporation is often used for transformation due to the extreme temperatures required for heat-shock and the lack of available viral vectors for transduction. In addition, the methylation pattern of each microbe must be determined to ensure self-recognition of transformed DNA. Other issues to consider are the availability or development of suicide or shuttle plasmid vectors and inducible or constitutive promoters.

Extreme thermophile genetics typically rely upon auxotrophic mutant parental strains because thermostable antibiotic markers are not available or the target organism is not susceptible to antibiotics. Uracil prototrophy selection in a uracil auxotrophic mutant strain, followed by counterselection using 5-fluororotic acid (5-FOA), is often the preferred strategy and has been used for *Sulfolobus* [7,8], *Thermococcus* [5,9], *Caldicellulosiruptor* [10,11] and *Pyrococcus* [12,13] species and most recently for *Thermotoga sp. RQ7* [14] and *Thermotoga maritima* [15]. Alternatively, agmatine prototrophy selection in agmatine auxotrophic mutants has been utilized in *S. islandicus* [16], *T. kodakarensis* [17] and *P. furiosus* [18] and a new counterselection system was described for *S. islandicus* [19] and *T. barophilus* [20] using an adenine phosphoribosyltransferase mutant and a purine analog (6-methylpurine).

Although not as prevalent, antibiotic markers have been used in extreme thermophiles. The *hmgA* (3-hydroxy-3-methylglutaryl coenzyme A reductase) gene has been successfully used as a marker in *P. furiosus* [21], *S. islandicus* [8,22], *T. barophilus* [9] and *T. kodakarensis* [23] for simvastatin selection. However, spontaneous mutations resulting in simvastatin resistant cells make this marker less appealing. The introduction of a heat-stable kanamycin marker in *C. bescii* increased the options for selectable markers [11] and additional heat-stable selectable markers are needed to alleviate the dependence on auxotrophic parent strains.

CRISPR/Cas targeted genome editing is a possible alternative for making markerless mutants. This RNA-guided system has been effectively employed in *S. islandicus* [24], leveraging its native CRISPR/Cas systems to generate genomic mutations. However, harnessing endogenous CRISPR systems for editing still requires a selectable marker for plasmid maintenance, so this technique can only be applied to organisms that have established transformation protocols. Beyond the generation of genomic mutants, the type III CRISPR/Cas system has also been used to target RNA transcripts and post-transcriptionally silence genes in *Sulfolobus solfataricus* [24], which may be a method to generate a desired phenotype without genomic modification.

A crucial aspect of metabolic engineering is the ability to regulate the expression of recombinant genes with inducible promoters, as the products of highly-expressed genes can produce unwanted side-products or have toxic effects. However, only a few inducible promoters have been developed, including the *araS* arabinose inducible promoter for *S. solfataricus* [25] and the *mal* maltose inducible promoter for *S. acidocaldarius* [26]. Other promising inducible promoters have been discovered recently, such as a xylose inducible promoter in *C. bescii* [27] and a fluoride inducible riboswitch in *T. kodakarensis* [28]. Concerns for developing novel inducible promoters are ensuring that the associated regulatory system is present and active at high temperatures. Depending on the downstream product, it may be important to minimize basal expression of the promoter in the absence of the inducer. Finally, the compound used for induction must not have an adverse effect on the cell. Simply finding native inducible promoters to use for genetic manipulation can alleviate many of these concerns. Furthermore, the expression level of the inducible promoter under different conditions must be evaluated.

Another tool that would be extremely useful but not widespread in extreme thermophile genetics are reporter systems. Reporters often aid in the study of microbial physiology but can also serve as measures of promoter strength, inducibility and basal expression activity. For example, β-galactosidase functioned as a reporter for promoter induction in *S. acidocaldarius*

when testing the maltose inducible promoter. A thermostable green fluorescent protein (GFP), another commonly used reporter in mesophilic model systems, has been utilized in *S. acidocaldarius* and the moderate thermophile *Parageobacillus thermoglucosidasius* (T_{opt} = 65 °C) with some success [29], but this reporter has not yet become a prevalent tool in extreme thermophiles. The heat-stability of the reporter protein, codon usage differences between the source and target organisms, and whether the systems signal is both sensitive and robust are issues to consider when developing new reporter systems.

Physiological Challenges and Opportunities

There are some unique challenges with extreme thermophile platforms. Recruiting heterologous genes that encode thermoactive, thermostable biocatalysts can be difficult and cofactor thermal stability and energy carrier management can influence how resources are allocated to different cellular processes. For example, NADH undergoes thermal decomposition to nicotinamide and ADP-ribose [30,31] and the salvage process consumes ATP, releasing AMP and pyrophosphate as final products (**Figure 1)** [31,32]. Deletion or disruption of the nicotinamide deaminase in both *T. kodakarensis* and *T. thermophilus* led to growth defects above 80°C, which were alleviated by supplementing the medium with nicotinic acid [31].

One potential mechanism for extreme thermophiles to cope with the ATP demand of NADH repair is to use kinases dependent on other phosphate carriers, such as ADP and pyrophosphate (PPi). ADP-dependent glucose/glucosamine kinases or fructose-6-phosphate kinases have been identified in *P. furiosus, P. horikoshii, T. litoralis,* and *T. kodakarensis* [33-35]. PPi-dependent kinases, while not exclusive to extreme thermophiles, can potentially offset energy expenditure of central metabolism by avoiding the use of ATP as a phosphate donor [36]. Recently, the structural determinants of using ATP or PPi as a phosphate donor have been determined based on comparative studies of myo-inositol kinases from *T. kodakarensis* and *T. maritima*, potentially allowing for engineering kinases to use PP instead of ATP [37].

Extremely thermophilic metabolic engineering platforms

Metabolic engineering successes with extreme thermophiles have been reported (**Table 2**, **Figure 2**). Although such developments are in ealy stages, they indicate the significant potential of these microorganisms for industrial biotechnology.

Caldicellulosiruptor bescii

Caldicellulosiruptor bescii (T_{opt} = 78°C) could potentially convert carbohydrates from plant biomass into fuels and chemicals, making it a promising candidate for use in Consolidated BioProcessing (CBP) [38]Multi-modular S-Layer Homology (SLH) domain hemicellulases associated with the cell envelope [39], and secreted, multi-modular cellulases encoded in a genomic region, referred to as the Glucan Degradation Locus (GDL), have been a focus for improving deconstruction of plant biomass [40]. The native enzyme-microbe synergism for lignocellulose degradation by *C. bescii* is highly optimized and could not be improved with exogenously added cellulase cocktails based on the *C. bescii* secretome [41]. Novel substrate-binding proteins, called tāpirins, have been examined in *Caldicellulosiruptor* species and overexpression of specific tāpirins genes might improve attachment to cellulosic moieties in plant biomass, potentially improving lignocellulose conversion [42,43].

Ethanol production directly from switchgrass has been in *C. bescii* by heterologous expression of the AdhE from *Clostridium thermocellum* at a titer of 0.6 g/L in a Δ*ldh* background [10]. Recently, expression of the *C. thermocellum* AdhE in a more genetically stable lineage of *C. bescii* along with co-expression of a ferredoxin NAD oxidoreductase improved ethanol titer to 3.5 g/L at 60°C for growth on crystalline cellulose, facilitated by an increase in available NADH [44,45]. While this represents a significant improvement in titer, the fermentation was performed at 60°C, due to the thermal instability of the AdhE, and generated byproducts, such as pyruvate and acetoin. This suggests the need for further genetic engineering and more stable biocatalysts to direct carbon and electron flux to the intended target.

Thermococcus kodakarensis

T. kodakarensis (T_{opt} = 85°C) grows on polysaccharides, starch, and pyruvate to produce hydrogen gas [46,47]. Recently, its ability to use chitin as a carbon source for H_2 production was recovered through overexpression of the endogenous chitinase, *N-,N'*-diacetylchitobiose catabolic pathway, and disruption of a glycolytic repressor with further improvements made by selecting for increased growth on chitin [48]. Additionally, verification of the open reading frame encoding the glucosamine kinase through gene disruption and recombinant protein expression provided a more complete picture about how chitin enters central metabolism [34]. While these chitinolytic strains have been focused on hydrogen production near the Thauer limit (4 mol H_2/mol glucose), they could serve as a chassis for production of industrial chemicals from an abundant, renewable carbon source. Production of the isoprenoid phytoene has also been demonstrated in *T. kodakarensis* via heterologous overexpression of the phytoene synthase from *S. acidocaldarius* [49]. Titers were improved by disrupting the acetyl-CoA synthase gene and adding an additional copy of the isoprenoid synthase.

Pyrococcus furiosus

Metabolically engineering of *P. furiosus* has been facilitated by the naturally competent, uracil auxotrophic, COM1 parent strain, which has been engineered to produce n-butanol, 3-hydroxypropionate, ethanol, and acetoin [4,50-52]. One of the major challenges to using *P. furiosus* as a metabolic engineering host is recruiting heterologous enzymes that are functional near its optimal growth temperature of 100°C. This issue can be addressed by growing *P. furiosus* to high cell densities near the optimal growth temperature, then lowering the temperature to the optima for the recombinant enzymes involved in product formation, since *P. furiosus* can retain significant metabolic activity at temperatures as low as 70°C [53]. This allows for the heterologous expression of genes from an expanded range of sources, namely,

microbes growing between 60 – 75°C. Recently, ethanol production has been demonstrated in *P. furiosus* via heterologous expression of various bifunctional alcohol dehydrogenases (AdhE) obtained from extremely and moderately thermophilic bacteria, albeit at low titers [54]. Ethanol production has also been demonstrated via ferredoxin dependent acetate reduction with recombinant expression of the acetaldehyde dehydrogenase (AdhA) from *Thermoanaerobacter* X514 at 70°C in conjunction with the native aldehyde oxidoreductase (AOR) of *P. furiosus* [55]. Deletion of the native AOR and expression of the AdhE and AdhA from *Thermoanaerobacter* strains resulted in a lower titer of ethanol, indicating that the AOR-AdhA pathway is more efficient for alcohol production; this is likely because *P. furiosus* manages its reducing equivalents with the native low potential ferredoxin rather than NAD(P) [54]. Moreover, the AOR-Adh pathway reduces other acids to alcohols besides acetate, potentially diversifying the products synthesized by extreme thermophiles. *P. furiosus* was engineered to use carbon monoxide as a source of energy through the expression of the 16-subunit CO dehydrogenase (Codh) complex from *T. onnurieneus* [50]. Interestingly, growth on CO helped the organism to utilize acetate for biosynthesis, which could be useful in converting initial fermentation products into higher value compounds.

Thermotoga maritima

Thermotoga species ferment sugars to make H_2 near the Thauer limit [56]. Utilization of *Thermotoga* as a metabolic engineering host has been limited primarily by the lack of a genetic system. However, genetic tools have recently been reported for *T. maritima*, facilitating potential engineering of hydrogen production [15]. A disruption of the lactate dehydrogenase generated a spontaneous mutant, which showed decreased sugar uptake and growth but increased hydrogen production over the Thauer limit. This is likely due to increased carbon flux through the oxidative pentose phosphate pathway, which can provide extra reducing equivalents for

hydrogen production with the growth defects due to mutations in an ABC maltose transporter, as revealed by whole-genome sequencing [57].

Sulfolobus sp.

Sulfolobus species use a broad range of carbon sources at low pH without carbon catabolite repression, potentially allowing this host to catabolize acid hydrolysates of lignocellulose [58]. Some species are able to grow chemoautotrophically on sulfur, from which sulfuric acid is produced as a major product. The physiology of *Sulfolobus* is well understood due to the range of available genetic tools and multi-omic studies that have been conducted [59]. Recently, gene deletion studies showed that pentose metabolism in *S. acidocaldarius* occurs through an aldolase-independent pathway [60] and the mechanism for formation of cyclopentane rings in its lipids [61]. Comparative multi–omic studies have also been performed for wild type *S. solfataricus* to investigate the physiological response of growth on different hexose sugars and ultimately provide experimental data to support metabolic models [62]. Although efforts to produce industrial chemicals in *Sulfolobus* have been limited by long doubling times and low biomass yields [59], there is room for further development of *Sulfolobus* sp. as metabolic engineering platforms.

Systems biology and metabolic modeling approaches for extreme thermophiles

Systems biology-based models are essential for optimizing metabolic engineering platforms and vary in complexity and scale, ranging from pathway analysis to genomic reconstructions of metabolism. These models provide a foundation for understanding host physiology and accelerate the development of industrial strains [63,64].

Maintaining optimal concentrations of enzymes in a multi-step pathway can reduce the metabolic burden of heterologous expression needed to produce a product of interest. Kinetic

models for such multi-step pathways are useful for optimizing pathway flux *in vitro*, as has been demonstrated for an extremely thermophilic n-butanol pathway [65], and for the 3-hydroxypropionate/4-hydroxybutryate (3-HB/4-HB) carbon fixation cycle from *Metallosphaera sedula* [66], which served as the basis for 3-hydroxypropionate formation in *P. furiosus*. Ultimately the development of *in vitro* kinetics models can allow for using exact intracellular concentrations to help debottleneck metabolic pathways.

Metabolomics and flux analysis can help validate metabolic models and ultimately elucidate global carbon flow and limitations in metabolic engineering efforts in extreme thermophiles [67]. In *C. bescii*, the global *rex* redox regulator was deleted and metabolomics experiments showed higher intracellular concentrations of organic acids and reducing equivalent availability, with a slightly higher ethanol yield, albeit at lower concentrations [68]. Recently, the combination of enzyme kinetics data from cell free extracts, deterministic and stochastic modeling, and metabolomics showed the robustness of the branched Entner-Doudoroff pathway in *S. solfataricus* to temperature and either branch deletion [69].

Transcriptomic analyses can elucidate global changes in gene expression, especially in response to changes in environment or genomic modifications. Batch and continuous culture were compared in *P. furiosus*, revealing differential regulation of over 200 genes as a function of metabolic mode [70]. In *C. saccharolyticus,* treatment of the carbon substrate can affect the transcriptome based on the availability of sugar substrates for the host, as solubilization of acid-treated switchgrass compared to low lignin lines of poplar created a significant change in the expression of genes related for xylan metabolism [71].

Genome reconstructions of metabolism are powerful tools for understanding the steady state metabolite profile based on genomic annotations for biological pathways [64]. Genome reconstructions have been reported for *T. maritima* [72] and *S. solfataricus* [58]. For *T. maritima*, a transcription factor network [73], metabolic reconstruction and a metabolism and expression model (ME) have been reported [74]. The latter ME model accounts for transcription and

translation demands, allowing for *in silico* prediction phenotype based on genotype [74,75]. These models have been limited primarily by their complexity, but new algorithms have been developed to overcome this [81]. Ultimately, metabolic models can provide a systematic approach for improving titers of chemicals in thermophiles by accounting for several of its unique physiological features.

Conclusions

Extreme thermophiles are emerging as promising hosts for industrial biotechnology catalyzed by the development and improvement of molecular genetic tools. This development has opened up strategies for using renewable resources to produce non-native fuels and chemicals. Extreme thermophile metabolic engineering faces the same challenges encountered with model mesophilic microorganisms, especially with achieving commercially-relevant productivities. If overcome, potential advantages with bioprocessing at elevated temperatures, especially minimal contamination issues, can be exploited.

Acknowledgments

This work was supported by grants to RMK and MWWA by the US National Science Foundation (CBET-1264052, CBET-1264053), to RMK by the US National Science Foundation (CBET-1802939), US Air Force Office of Scientific Research (AFOSR) (FA9550-13-1-0236, FA9550-17-1-0268), US Department of Agriculture (NIFA 2018-67021-27716), and to MWWA by the US Department of Energy (DE-FG05-95ER20175). CTS and JRC acknowledge support from a US Department of Education GAANN Fellowship (P200A140020, P200A160061).

Table 1: Current Status of Genetic Tools for Extreme Thermophiles											
	***pyrEF*/5-FOA**	***trpE*/6-methylpurine**	***apt*/6-methylpurine**	**Agmatine Prototrophy**	**Lactose Prototrophy**	**Arginine/Citrulline**	**Kanamycin**	**Simvastatin**	**Hygromycin**	**Naturally Competent**	**References**
Organism											
Caldicellulosiruptor											
Caldicellulosiruptor bescii	X						X				[11]
Pyrococci											
Pyrococcus abyssi	X										[13]
Pyrococcus furiosus	X							X		X	[4,12,21]
Sulfolobi											
Sulfolobus acidocaldarius	X										[7]
Sulfolobus islandicus	X		X	X				X			[8,16,19,22]
Sulfolobus solfataricus					X				X		[76,77]
Thermococci											
Thermococcus barophilus	X		X					X			[9,20]
Thermococcus kodakarensis	X	X		X		X		X		X	[5,17,23,78]
Thermotoga											
Thermotoga maritima	X						X			X*	[15]
Thermotoga sp. RQ7	X						X			X	[6,14,79]

*Conflicting reports

Squares highlighted in blue denote systems reported after 2016

	Table 2: Industrial Chemical Products from Metabolically Engineered Extreme Thermophiles					
Product	**Host**	**Carbon Source**	**Temperature**	**Titer**	**Description**	**Ref.**
Hydrogen	*Thermotoga maritima*	Maltose	80 °C	15 mM	Ldh disruption with spontaneous mutant, H_2 production above Thauer limit, titer reached after 5 hours	[57]
	Thermococcus kodakarensis	Chitin	85 °C	30 mM	Restoration of chitinolytic phenotype with genetic and adaptive engineering	[48]
Ethanol	*Pyrococcus furiosus*	Maltose	70 °C	1.5 g/L (32 mM)	Major side product from butanol pathway insertion	[51]
	Pyrococcus furiosus	Maltose	70 °C	0.92 g/L (20 mM)	Acetate reincorporation by native acetate oxidoreductase and AdhA expression	[55]
	Pyrococcus furiosus	Maltose	70 °C	1.6 g/L (35 mM)	Deletion of acetolactate synthase	[52]
	Caldicellulosiruptor bescii	Crystalline Cellulose	60 °C	3.5 g/L (76 mM)	Expression of *Clostridium thermocellum* AdhE in stable background with coexpression of reduced ferredoxin:NADH oxidoreductase	[44]
	Caldicellulosiruptor bescii	Switchgrass	65 °C	0.6 g/L 12.8 mM	Expression of *Clostridium thermocellum* AdhE	[10]
Phytoene	*Thermococcus kodakarensis*	Yeast extract and tryptone	72 °C	2.6 mg/L (5 µM)	Expression of multiple copies of phytoene synthase from *Sulfolobus solfataricus*	[49]
Acetoin	*Pyrococcus furiosus*	Maltose	70 °C	0.53 g/L (6 mM)	Native pathway, deletion of this pathway improved ethanol titer	[52]
3-HP	*Pyrococcus furiosus*	Maltose	70 °C	0.34 g/L (3.7 mM)	Expression of genes from 3-HP/4-HB carbon fixation cycle from *Metallosphaera sedula*	[80]
Butanol	*Pyrococcus furiosus*	Maltose	70 °C	0.070 g/L (0.95 mM)	Hybrid pathway from 3 moderate thermophiles, pathway also reported *in vitro* butanol production	[51]

Figure Captions

Figure 1: NAD^+ salvage pathway. Thermal decomposition of NAD^+ generates ADP-ribose and nicotinamide, which are regenerated through a multistep pathway. Energy input steps are shown with silver arrows. Deamidation of nicotinamide (shown in red) was demonstrated to be essential for growth at higher temperature for *T. kodakarensis* and *T. thermophilus*. Adapted from [38,39].

Figure 2: Pathways used in metabolic engineering of extreme thermophiles. Host, final titer, and production temperature are reported below the products (blue rectangles).

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Graphical Abstract

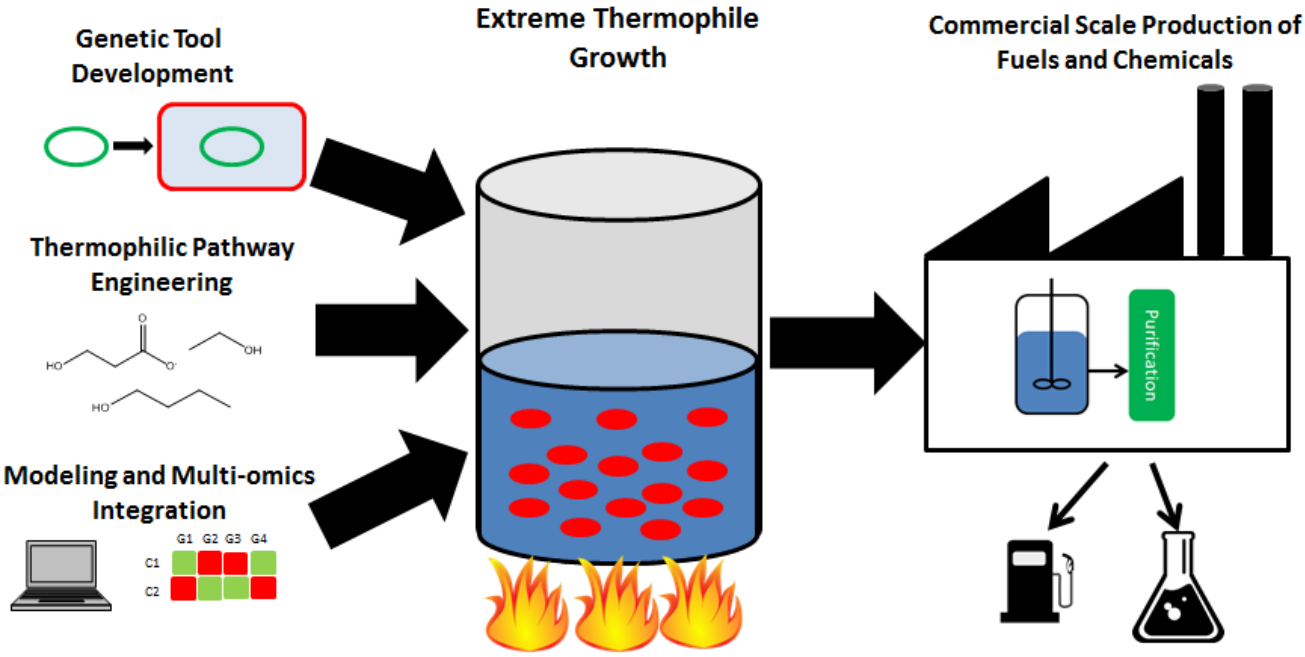

Figure 1: NAD Salvage Pathway

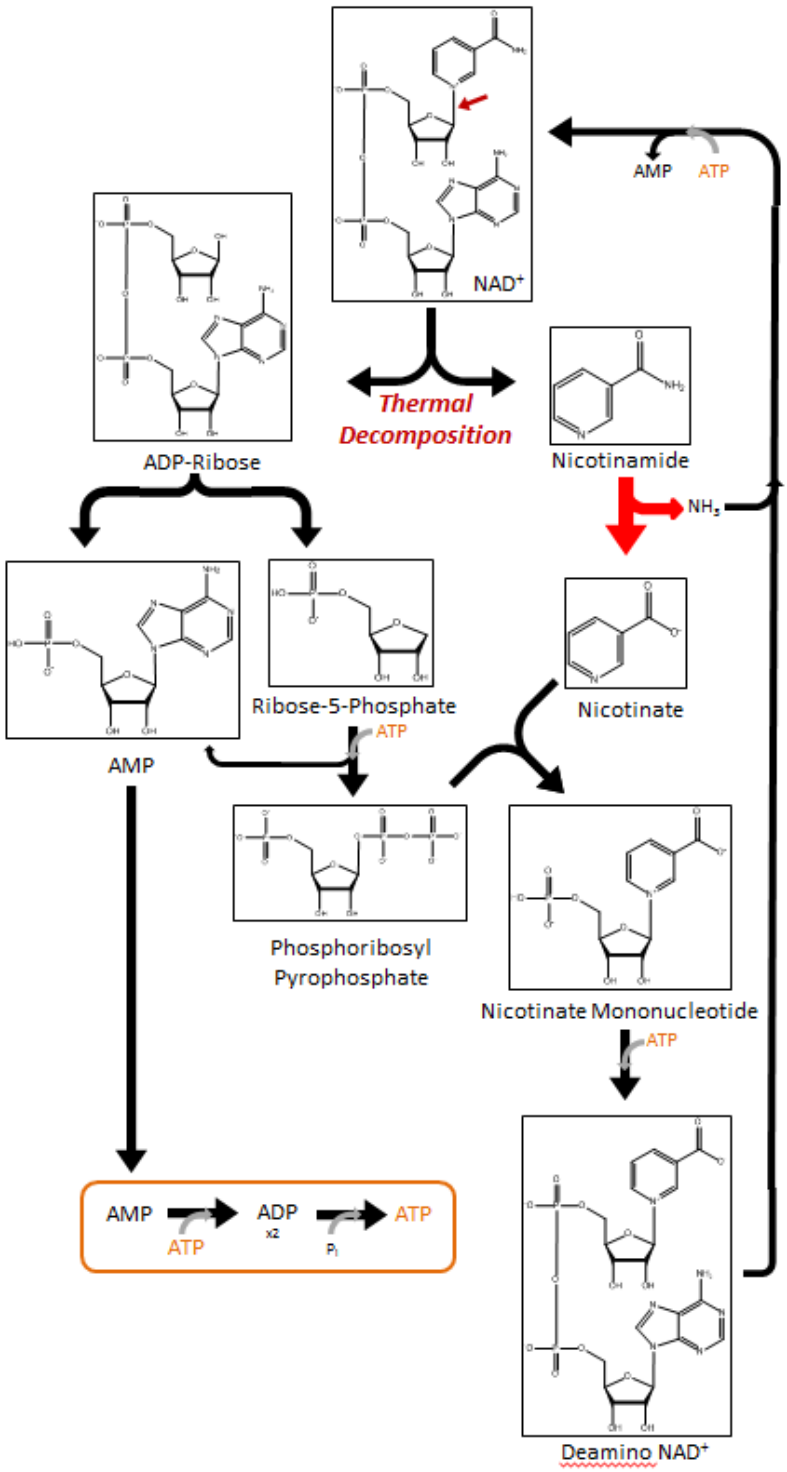

Figure 2: Pathways used to date in metabolic engineering of extreme thermophiles