Research review paper

Dissecting and engineering metabolic and regulatory networks of thermophilic bacteria for biofuel production

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ABSTRACT

Interest in thermophilic bacteria as live-cell catalysts in biofuel and biochemical industry has surged in recent years, due to their tolerance of high temperature and wide spectrum of carbon-sources that include cellulose. However, their direct employment as microbial cellular factories in the highly demanding industrial conditions has been hindered by uncompetitive biofuel productivity, relatively low tolerance to solvent and osmotic stresses, and limitation in genome engineering tools. In this work we review recent advances in dissecting and engineering the metabolic and regulatory networks of thermophilic bacteria for improving the traits of key interest in biofuel industry: cellulose degradation, pentose–hexose co-utilization, and tolerance of thermal, osmotic, and solvent stresses. Moreover, new technologies enabling more efficient genetic engineering of thermophiles were discussed, such as improved electroporation, ultrasound-mediated DNA delivery, as well as thermo-stable plasmids and functional selection systems. Expanded applications of such technological advancements in thermophilic microbes promise to substantiate a synthetic biology perspective, where functional parts, module, chassis, cells and consortia were modularly designed and rationally assembled for the many missions at industry and nature that demand the extraordinary talents of these extremophiles.

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1. Introduction

Temperature is one of the most important environmental parameters that affect microbial growth and distribution in our biosphere (Cava et al., 2009; Herbert, 1992). Although most contemporary life-forms are found at a narrow range of 24–40 °C (i.e., the mesophiles), the thermophiles, mostly of bacteria, archaea and fungi, thrive under optimal temperature of ≥50 °C (Brock, 1986; Oberson et al., 1999; Wiegel and Adams, 2003). Research during the last three decades revealed that the thermophiles play profound roles not only in the ecology, but also in the evolution of our biosphere, as primordial lives on earth are believed to be...
thermophilic (Cava et al., 2009; Wiegel and Adams, 2003). Furthermore, these extraordinary life-forms have found extensive applications in bioindustry (Blumer-Schuelle et al., 2008; Cava et al., 2009; Taylor et al., 2009).

There are a number of potential advantages in operating bioprocesses under high temperature (> 50 °C) (Georgieva et al., 2008; Taylor et al., 2009). First, high temperature accelerates the chemical reaction rate based on the Arrhenius equation (Connors, 1990). For instance, cellulases from the mesophile Trichoderma reesei (optimal growth at 30 °C) exhibit maximal enzymatic activity at 50 °C (Georgieva et al., 2008; Xu et al., 2010). Another example is Family 9 cellulases from the thermophile Clostridium thermocellum, whose enzyme catalytic activities are similar to their homologs in the mesophile Clostridium cellulolyticum up to 60 °C, but are 1.9 times higher at elevated temperature (80 °C) (Mingardon et al., 2011). Second, high temperature can avoid or minimize microbial contamination, which is a significant problem in many industrial processes. Addition of antibiotics, which can introduce significant costs and negative environmental consequence, is usually required to prevent, eliminate, or contain such biological contaminations (Skinner and Leathers, 2004; Taylor et al., 2009). Third, high temperature helps to reduce energy input. It promotes better solubility and efficient mixtures of substrate, and avoids cooling between the pretreatment/hydrolysis of cellulosic feedstock (which usually demands heating) and sugar fermentation (which is usually mesophilic; Lynd et al., 2008; Taylor et al., 2009; Turner et al., 2007)). Finally, high temperature facilitates downstream product recovery. Current techniques for solvent recovery mainly include distillation (gas stripping and steam stripping) and permeation membrane separation. As aqueous ethanol readily vaporizes at over 50 °C (Taylor et al., 2009), energy input for solvent recovery via steam stripping can be reduced by half when the operating temperature is shifted from 35 °C to 65 °C (Vane and Alvarez, 2008).

Therefore, besides their contribution as a valuable source of thermostable enzymes such as hydrolases (e.g., proteases, glycosidases and cellulase), DNA polymerases (e.g., Taq) and alcohol dehydrogenases (Atomi et al., 2011; Cava et al., 2009), the thermophiles are promising microbial cellular factories. For example, thermophilic Gram-positive anaerobes (TGPAs), such as certain Thermoaerobacter and Clostridium species, are of interest in producing cellulosic solvents (e.g., ethanol, butanol and isopropanol) under a Consolidated Bioprocessing (CBP) scheme, due to their wide spectrum of carbon-sources, co-utilization of pentose and hexose and ability to tolerate pH fluctuation (Fig. 1) (Chang and Yao, 2011; Demain et al., 2005; Lin et al., 2011; Taylor et al., 2009). Moreover, TGPAs, such as Caldicellulosiruptor saccharolyticus, are capable of producing hydrogen from lignocellulosic and waste materials (Willquist et al., 2010).

A number of mechanisms are thought to underlie the adaptation of the thermophiles to their extraordinary growth temperature (Kumar and Nussinov, 2001; Moat et al., 2002; Trivedi et al., 2005). First, the sequence and structure of nucleic acids in the thermophiles exhibit unique features as compared to the mesophiles. For example, at the DNA sequence level, high CG-content in the coding regions and higher frequencies of purine–purine and pyrimidine–pyrimidine were reported for the thermophiles (Trivedi et al., 2005). At the structure level, features of the thermophiles include methylation of nucleotides at different positions, the presence of reverse gyrase, and association with histone/histone like proteins and cations (e.g., K⁺), etc. (Trivedi et al., 2005). These features should contribute to the thermal stability of the genome. Second, structural lipids found in thermophilic organisms were of higher melting points than those in the mesophiles, suggesting that the melting temperature of major cellular lipid components might underlie the upper limit of growth temperature (Moat et al., 2002). Third, metabolic rates can be higher in the thermophiles which might facilitate rapid regeneration of those cellular components denatured by heat (Moat et al., 2002). Finally, proper functions of protein are maintained under high temperature, through subtle changes in protein structure and alterations in hydrogen bonding, hydrophobic interactions and other noncovalent activities (England et al., 2003; Kumar and Nussinov, 2001; Moat et al., 2002).

These genetic footprints left by thermo-adaptation might have profound implications in the cellular adaptation strategy to environmental stresses (Boor, 2006; Moat et al., 2002). The temperature sensitivity of many genetic mutations suggests that mutations that can be tolerated mesophilically might become lethal in the thermophiles (Drake, 2009). Thus thermophiles might be more sensitive than the mesophiles to the numerous environmental stresses typically encountered during the demanding industrial bioprocess. As a result, several disadvantages have been hindering the direct industrial applications of most wild-type thermophilic bacteria. (i) Continuous solvent production is highly

![Fig. 1. Advantages and disadvantages of the thermophiles for the industrial productions of fuels and chemicals. C5: pentose; C6: hexose; S: solvents.](image-url)
dependent on both efficient and simultaneous utilization of all di- and mono-saccharides released from lignocelluloses. However, high concentrations of carbon substrates often inhibit the fermentation process of the thermophiles (Argyros et al., 2011; Lin et al., 2011). (ii) Thermophilic bacterial cells are typically sensitive to the end product ethanol or butanol, and tolerance levels >40 g/L are rare in wild-type strains (Taylor et al., 2009). (iii) Typically, low product yield was observed, probably due to mixed acid fermentation (e.g., acetate and lactate) and less tolerance of end-products (Shaw et al., 2008; Taylor et al., 2009).

Removing these roadblocks in exploiting the thermophiles as cellular factory requires a thorough understanding of the gene networks underlying these traits for the thermophiles. In addition, widely accessible and high-throughput genetic systems for the screening and engineering of thermophilic bacterial strains are essential.

2. Carbohydrate utilization in the thermophiles

Thermophilic bacteria can utilize a remarkable variety of carbohyd- rates, from complex and incalcitrant polysaccharides (e.g., cellulose) to mono- and di-saccharides (Fig. 1) (Blumer-Schuette et al., 2008; Taylor et al., 2009). However, the breadth and patterns of edible poly- saccharides can vary, which are potentially linked to their distinct ecological niches and different optimal growth temperatures (T_{opt}) (Blumer-Schuette et al., 2008). For example, several hyperthermo- philic marine bacteria (T_{opt} ≥ 80 °C, e.g., Thermogalactonema) can catabo- lize glucans and hemicellulose (Blumer-Schuette et al., 2008), yet none of them can efficiently utilize crystalline cellulose, whereas many terrestrial thermophilic bacteria are cellulolytic. Among cellulolytic thermophiles, those with upper growth-temperature limit near 78 °C frequently degrade cellulose via “free-acting” cellulases, as repre- sented by the two genera Anaerocellulum (Anaerocellulum thermophilum, T_{opt} 75 °C) and Caldcellulosiruptor (Caldcellulosiruptor kristjanssonii, T_{opt} 78 °C; Caldcellulosiruptor besici, T_{opt} 80 °C) (Blumer-Schuette et al., 2008; Demain et al., 2011). In contrast, those with lower optimal temperature often produce cellulosomes to degrade crystalline cellulose (e.g., C. thermocellum (T_{opt} 60 °C)) (Demain et al., 2005).

Comparison of eight Caldcellulosiruptor genomes revealed significant inter-genome differences in glycoside hydrolase inventories and the number of carbohydrate transporters, even though their central metabolism pathways are highly conserved, indicating varied capacity in plant biomass degradation among members of Caldcellulosiruptor (Blumer-Schuette et al., 2011). Intriguingly, C. bescii is not only able to degrade various polysaccharides and unprocessed plant biomass, but also capable of degrading cellulose and xylan simultaneously (Demain et al., 2011). Genomic, transcriptomic and proteomic analysis revealed its several features in polysaccharide degradation (Blumer-Schuette et al., 2011; Dam et al., 2011), which included multi-modal, multi-functional carbohydrate-active (CAzy) protein genes organized into one large functional gene cluster, high dosage of certain CAZymes mediated by gene duplication, and acquisition of CAzy genes and ABC transporters via lateral gene transfer (LGT). Furthermore, instead of employing the cellulosomes, C. bescii does not encode dockerins, and flexibly produces combinations of “free-acting” cellulases in response to various insoluble polysaccharides.

Cellulosomes are large extracellular celulolytic enzyme complexes and consist of nonenzymatic scaffolding proteins and cellulosomes, which are produced by cellulolytic mesophilic and thermophilic anaer- obes such as Clostridium, Acetivibrio, Bacteroides and Ruminococcus (Bayer et al., 2008; Demain et al., 2005; Doi et al., 2003). One model thermophilic cellulosome-producing bacteria is C. thermocellum (Bayer et al., 2008; Dam et al., 2011; Demain et al., 2005). The components, organizations and regulatory modes of cellulosomes in C. thermocellum appear to be distinct from those in mesophilic cellulosome-producing bacteria (e.g., Clostridium cellulovorans and C. cellulolyticum). In the C. thermocellum paradigm, cellulosomes contain a primary (enzyme-integrating) scaffoldin and anchoring scaffoldins, which form scaffoldin gene clusters (Demain et al., 2005). However, in mesophilic C. cellulovorans and C. cellulolyticum, only a primary scaffoldin without anchoring scaffoldins is present. Moreover, this scaffoldin gene is usually located upstream of a series of genes coding for cellulosomal enzymes, as opposed to the “scaffoldin gene cluster” in C. thermocellum (Bayer et al., 2008; Demain et al., 2005; Doi et al., 2003).

In C. cellulovorans and C. cellulolyticum, cellulosomal genes (nine or even larger) are found as a large operon, suggesting strict co-expression of these genes (Doi et al., 2003). However, in C. thermocellum (Demain et al., 2005), such genes (e.g., cellulase and xylanase genes) are present as individually located genes scattering over the chromosome or as small cellulosomal gene clusters (six or less genes) (Demain et al., 2005). Thus it is possible that the expression patterns of these cellulosomal genes might be relatively independent and under the reg- ulation of multiple regulators, in contrast to the likely fewer regulators in C. cellulovorans and C. cellulolyticum (Doi et al., 2003). This suggests that thermophiles might be able to precisely tune the active portfolio of cellulosome components in response to different growth conditions. With an increased number of cellulosolytic Clostridium genomes being sequenced (Feinberg et al., 2011; Hemme et al., 2010; Roberts et al., 2010), the structure and regulation of the “cellulose degradomes”, i.e., the genome-wide metabolic and regulatory networks underpinning cellulose degradation, will be emerging.

For monosaccharide utilization, desirable features were also found in certain thermophiles. Hextose and pentose co-utilization is a highly valuable trait among biofuel-producing microbes. However, most known mesophilic cellulose degrader or ethanologenes either are unable to ferment pentoses to ethanol (e.g., Saccharomyces cerevisiae and Zymomonas mobilis (Lynd et al., 2002)) or prefer hexoses over pentoses (e.g., mesophilic Clostridium (Servinsky et al., 2010; Tracy et al., 2012), Escherichia coli (Deutscher, 2008) and Bacillus subtilis (Gorke and Stulke, 2008), due to a mechanism called carbon catabolite repression (CCR) (Gorke and Stulke, 2008) (Fig. 3). Interestingly, CCR appears to be absent in many thermophiles, e.g., C. saccharolyticus (Vanfossen et al., 2009), Thermoanaerobacter sp. X514 (Lin et al., 2011), Thermoanaerobacter ethanolicus 39E (Jones et al., 2002) and Thermoanaerobacterium saccharolyticum JW/SL-YS485 (Shaw et al., 2008). In other words, these thermophiles can ferment hextose and pentose for biofuels in a simultaneous and unbiased manner.

Genome comparison of multiple Thermoanaerobacter strains, coupled with experimental analyses, revealed that Thermoanaerobacter sp. X514 was endowed with additional xylose transporters (which confer its ability to grow at lower xylose concentrations than T. ethanolicus 39E) and a modified pentose catabolism that mediates greater abso- lute carbon flux from xylose and thus likely increases ethanol produc- tion (Hemme et al., 2011). Moreover, genome-wide regulatory networks of the “thermophilic” glycocon in Thermoanaerobacter revealed the mechanism of pentose and hexose co-utilization, which was rationally exploited for enhanced ethanol production (Lin et al., 2011) (Fig. 2). In this gene network of glycocon, gene modules related to fructose, cellobiose and hexose are “standalone”, without inter-module interactions, suggesting a certain degree of independence between hexose- and pentose-utilization pathways. Moreover, transcriptional choreographies of the Thermoanaerobacter glycocon along the bacterial growth course revealed the cooperating nature of pentose- and hexose-catabolism in this organism. Specifically, glucose accelerates xylose utilization via activating xylo in transport and catabolism genes, whereas xylose maintains and extends coenzyme activities and ion metabolism which delay cell lysis. This intriguing mode of monosaccharide catabolism can be interpreted at the regu- latory level (Fig. 3; (Lin et al., 2011)). In the mesophiles such as E. coli and B. subtilis, the xylose utilization is regulated by XylR (Deutscher et al., 2006; Song and Park, 1997). In B. subtilis, in the absence of xylose, XylR, the xyl-loci repressor, binds to the xyl operators O₁ and O₂ to block
xylose transcription. When xylose is present, xylose interacts with XylR, which abolishes the binding between XylR and xyl operators and induces the expression of xyl loci. When glucose and xylose are both present, glucose induces phosphorylation of Hpr and the co-repressor Crh (a novel Hpr-like protein also involved in CCR) and activates these two trans-acting factors. The activated Hpr and Crh bind the catabolite responsive element (CRE, located within the coding sequence of xylA), thus blocking xylose from being consumed until glucose is depleted (Dahl and Hillen, 1995; Gorke and Stulke, 2008). In *Thermoanaerobacter*, however, both hexose- and pentose-transport systems appear to be regulated by BglGs (rather than by XylR for pentose utilization in many other bacteria) (Fux et al., 2003; Lin et al., 2011): one BglG (Teth5140269) positively regulates xylose ABC-transporters, while another BglG (Teth5140414) activates glucose-specific PTS transporters. Such a regulatory mode might underlie the co-utilization of pentose and hexose in this and related organisms (Fig. 3).

3. Short-term stress (shock) responses in the thermophiles

Environmental perturbations are unavoidably encountered in the actual industrial bioprocesses, including those producing biofuels. They lead to the exposure of industrial strains (i.e., the thermophiles) to, usually simultaneously, a wide variety of environmental stresses, e.g., high concentrations of metabolites and substrates, extreme pH and exceedingly high oxygen levels. Thus, understanding how industrial strains sense and respond to impromptu changes and perpetual fluctuations in the environmental conditions (i.e., the ‘shock’) is crucial. The thermophiles can tolerate high temperature, which is a thermal stress for the mesophiles. Hence, these extremophiles probably mobilize specific mechanisms to cope with variable environmental stresses.

3.1. The thermophiles employ distinct mechanisms for thermal stress responses

Heat responses of mesophilic bacteria which grows optimally at 24–40 °C were intensively investigated (Boor, 2006; Chhabra et al., 2006; Moat et al., 2002; Ron, 2006). They employ alternative sigma factor, σ32, to induce a large array of heat shock proteins (HrcA–GrpE–DnaJ–DnaK, GroEL–GroES (the Group I complexes) and ATP-dependent proteases) that protect cells from damage (Moat et al., 2002). However, the thermophiles apparently mobilize distinct genes (or similar genes yet with different expression patterns) to cope with heat shock, including a global σ factor, constitutively expressed GroEL, ATPases, Group III chaperonins, and genes involved in central carbon metabolism pathways (Li et al., 2010; Moat et al., 2002; Pysz et al., 2004; Techtmann and Robb, 2010; Wang et al., 2012). In the thermophile *Thermotoga maritima*, the ortholog of this major heat-shock σ factor is absent and a global σ factor regulates heat-shock genes, which was experimentally validated (Pysz et al., 2004). It appears that these heat shock genes are constitutively expressed at T°opt, which might be the mechanism of thermal adaptation for these thermophiles. Notably, among these heat shock genes, the constitutively expressed heat shock protein 60 (Hsp60, i.e., the GroEL-equivalent in prokaryotes) at T°opt, is the hallmark of the heat-shock response in the thermophiles, probably due to its specific regulatory mechanism (Pysz et al., 2004). In *T. maritima*, an internal promoter specifically regulates the transcriptional activity of groEL.
(Pysz et al., 2004), which is a mechanism not yet reported in the mesophiles. In addition, in contrast to up-regulation of the vast ATP-dependent proteases (e.g., Lon, ClpA, ClpQ, ClpP, ClpX, ClpY and ClpB) in the mesophiles such as *E. coli* (Ron, 2006), only several ATPases (ClpC-1 and ClpC-2) are induced during heat shock in *T. maritima* (Pysz et al., 2004), suggesting that their strategies responding to thermal stress can be quite different. Furthermore, in thermophilic bacteria (e.g., *Carboxydothermus hydrogenoformans*, *Geobacillus* sp., *Desulfuris audaxviator* and *Thermosinus carboxydivorans*), Group III chaperonins were found which are distinct from the conventional Group I chaperonins present in bacteria (Saibil, 2008). These chaperonins, which are mostly located in the operon with DnaK and its cochaperones (DnaJ and GrpE), can refold denatured proteins in a GroES-independent manner (Techtmann and Robb, 2010). Interestingly, this lineage is structurally similar to the Group II chaperonins which are found in archaea and eukaryotes (Large et al., 2009; Zhang et al., 2010), representing an ancient LGT from an archaeon into an early Firmicute lineage (Techtmann and Robb, 2010). Beyond these stress response genes, a number of enzymes involved in central carbon metabolism pathways are activated at elevated temperature in the thermophiles. For example, in *Thermus thermophilus*, up-regulation of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was found to be related to thermoadaptation (Li et al., 2010). Similar observations were reported in *T. maritima*, where GAPDH and pyruvate synthase (PFOR) were induced at above-optimum temperature (Wang et al., 2012). Thus the up-regulation of enzymes in central carbon metabolism might be one factor underlying thermoadaptation in the thermophiles.

### 3.2. The thermophiles employ specific solutes to resist osmotic stress

The concentration of solutes (e.g., salts, ions and metabolites) plays a critical role in microbial growth. A sudden change of the solute concentration around the cells would cause cellular exposure to osmotic stress, due to a rapid fluctuation in the movement of water across their cellular membranes (Csonka, 1989). Most microbes prefer growth under relatively low osmolality (Moat et al., 2002). However, in industrial production, rapid accumulation and abrupt fluctuation of metabolites and solutes can be unavoidable, thus understanding how the thermophiles resist to osmotic shock is essential to engineering their robustness. In general, bacteria appear to employ a largely similar strategy in response to high osmolality: the accumulation of compatible solutes (i.e., small organic osmolytes (Kurz, 2008)) either by uptake from the medium or by de novo synthesis (Csonka, 1989; Empadinhas and da Costa, 2006). Interestingly, the kinds of such compatible solutes mobilized by bacteria appear to be related to the *T*<sub>opt</sub> of the organism. For instance, in the mesophiles, an increase in the flux of K<sup>+</sup> ion is the earliest response to osmotic upshock, and then major anionic compounds (glutamate) are synthesized and accumulated; after the decline of K<sup>+</sup>/glutamate level, trehalose level rises for osmoprotection (Moat et al., 2002). However, most thermophiles synthesize mannosylglycerate (MG) and/or Glucosylglycerate (GG) as the primary osmolyte, which are lipid molecules not commonly found in the mesophiles (Alarico et al., 2007; Empadinhas and da Costa, 2006; Neves et al., 2005; Santos and da Costa, 2002). Moreover, the observed distribution of MG synthesis genes throughout thermophilic and hyperthermophilic prokaryotes

![Fig. 3. Molecular mechanisms for the co-utilization of pentose and hexose in certain thermophilic bacteria.](image-url)
suggests that MG might contribute to the thermal adaptation of these organisms (Borges et al., 2002; Empadinhas and da Costa, 2006). In fact, MG was found to be one of the strongest thermoprotectants against thermal denaturation of enzymes such as lactate dehydrogenase and glucose oxidase (Borges et al., 2002).

3.3. The thermophiles are sensitive to solvent stress

Industrial strains produce solvent products through fermentation, however they can be sensitive to their own solvent products (Taylor et al., 2008, 2009), which reduce cell vitality, impair membrane integrity, inhibit enzymes and/or perturb intracellular pH balance (Taylor et al., 2008; Timmons et al., 2009). Interestingly, the sensitivity to solvent stress is likely linked to temperature, as thermophiles can be less tolerant to a high-level of solvents such as ethanol than the mesophilic ethanologen such as Z. mobilis and S. cerevisiae (Timmons et al., 2009). Even among the thermophiles, ethanol tolerance is temperature dependent. In Clostridium thermohydrodsulphuricum, an ethanol tolerant mutant can grow under up to 8.0% (wt/vol) ethanol at 45 °C, but only under up to 3.3% (wt/vol) at 68 °C (Lovitt et al., 1984).

The cellular responses of the mesophiles and the thermophiles to solvent stress exhibit different features. In mesophiles, the general solvent responses involve increased membrane fluidity, solvent exclusion systems, energy-dependent efflux pumps of the resistance-nodulation-cell division (RND) family, stress−response genes (sosX, marA and robA encoding DNA-binding proteins/transcriptional activators), mannose transporter of the phosphotransferase system (manXYZ), ATPase, heat shock proteins (GroESL and redox balance maintenance (Ma and Liu, 2010; Okochi et al., 2007; Ramos et al., 2002; Rutherford et al., 2010; Taylor et al., 2008; Tomas et al., 2004). In thermophilic bacteria, however, the modes of solvent-induced membrane alterations are different from those of the mesophiles. Ethanol-adapted C. thermocellum increased membrane rigidity to counter-act the fluidizing effect of ethanol (Timmons et al., 2009), while the mesophilic S. cerevisiae increased membrane fluidity via increased unsaturated/saturated fatty acid ratio in membranes (Stanley et al., 2010).

Moreover, in the thermophiles, alcohol dehydrogenase (adh) plays a key role in resisting ethanol stress, although the mechanisms can vary. In T. ethanolicus 39E, a strain tolerating 8% ethanol lacks primary adh (associated with ethanol consumption) (Burdeette et al., 2002). In C. thermocellum, a mutated adhE where co-factor specificity was shifted from NADH to NADPH endows a wild-type strain with tolerance to 40 g/L ethanol (Brown et al., 2011). However, similar adh functions were not observed in the mesophiles such as S. cerevisiae (Alper et al., 2006) and E. coli (Chen et al., 2011).

Furthermore, although the transcriptional activation of the Hsps is one prominent and shared feature of the general shock−response in mesophiles such as E. coli and S. cerevisiae (Rutherford et al., 2010; Stanley et al., 2010), so far no studies have reported in the thermophiles such roles of Hsps in the cellular response to solvent stress.

These findings suggested that successful engineering of thermophilic bacteria for enhanced solvent tolerance might require new strategies and targets derived from thermophile-specific metabolic and regulatory networks.

4. Adaptive evolution in the thermophiles

Shock response is a transient reprogramming of metabolic and regulatory networks to extend organismal survival under stresses (Stanley et al., 2010). Distinct from the shock response, adaptive evolution (or, “tolerance”) typically starts from an environmental change and results in genetically inheritable adaptation (Blount et al., 2012; Lenski et al., 2003). Shock responses do not necessarily lead to tolerance, as the latter typically requires generations of selection for genetic changes to take place in a population (Ma and Liu, 2010). Hence, understanding the process of adaptive evolution can be valuable to rationally engineering the stress tolerance of industrial strains (Fig. 2).

As a universal theme of life on our planet (Barrick et al., 2009; Lenski et al., 2003; Wiegand and Adams, 2003), adaptive evolution remains poorly understood for the lives under high temperature, although microevolution under moderate temperature has been intensively studied (Blount et al., 2012). Key questions remain unanswered: for example, whether and how the evolution process was shaped by temperature? Do the thermophiles undergo a distinct adaptation program? Can we modulate the co-evolution of multiple desirable traits in the thermophiles?

4.1. The thermophiles appear to exhibit lower mutation rates

Genome mutations result from and reflect the adaptability of organisms to the changing environment. Evolutionary success of bacteria relies on their mutations, whose rate might be constantly fine-tuned by mutator alleles (controlling the fidelity of genome replication and repair) at different levels under disreputable environments (Barrick et al., 2009; Bronham, 2009; Denamur and Matic, 2006). Hence, the mutation rate is determined by the equilibrium between deleteriousness of mutations and costs of further reducing mutation rates (Drake, 2009). Interestingly, the mutation rate seems linked to microbial adaptation to the thermophilic environment, as thermophilic bacteria appear to display lower mutation rates than mesophiles under optimal growth conditions (Drake, 2008). Drake explained this phenomenon by organismal adaption to avoid deleterious mutations at high temperature (Drake, 2008). It was often observed that the most common class of mutations is related to temperature sensitivity; moreover many missense mutations especially those concerning protein folding are well tolerated at the standard growth temperature, but become much more deleterious, often to the point of lethality, at a temperature only 5–10 °C higher (Drake, 2009). However, experimental supports for this postulation have been scarce and elusive.

One of the few previous attempts to measure the mutation rate of the thermophiles was conducted under optimal growth condition based on mutation reporter gene pyrEF using the CT (chain-termination) method (Drake, 2009), which is a simple, rapid and cost-effective method. However, there are several drawbacks associated with this approach, including massive mutational hotspots scattering over the genomes (variation of mutation rates among different sites), the numeric minority of CT mutations among all mutations and missing A:T→G:C mutations, all of which might lead to reduced accuracy in mutation rate measurement (Drake, 2009). Hence, whole-genome sequencing is preferable for accurate assessment of the mutation rate. In fact, no mutation rates for thermophilic organisms have been experimentally measured based on genome-wide approaches, despite that the mutation rates of many mesophiles have been measured using whole-genome sequencing (Barrick et al., 2009; Gundry and Vijg, 2012). Comparison of genome-wide mutation rates between the thermophiles and the mesophiles, under optimal growth or in a variety of stressed conditions, promised to bring in another dimension of understanding on how DNA and cellular lives adapt to extreme environments.

4.2. Co-evolution of traits

Although its molecular mechanism is not yet well understood, adaptive evolution has been a widely practiced strategy for selection and engineering of economically valuable traits for strain development in the biotechnology industry (Atsumi et al., 2010; Brown et al., 2011; Minty et al., 2011). One such example is the tolerance to solvent, which typically requires improvement in wild-type ethanologens that include many thermophiles (Dunlop, 2011; Taylor et al., 2009). This trait can be improved by adaptive evolution, via sequential transfers of cultures under incremental concentrations of exogenous solvents...
(Atsumi et al., 2010; Brown et al., 2011). However, studies such as those in S. cerevisiae (Goodarzi et al., 2010) and E. coli (Goodarzi et al., 2010) revealed frequently the negative correlation between ethanol tolerance and ethanol productivity, both of which are traits of crucial interest in improving overall bioprocess productivity. The thermophiles are probably no exceptions. In a recent report, a C. thermocellum strain with elevated tolerance to ethanol produced less ethanol (Brown et al., 2011).

In mesophiles, several strategies were reported to engineer the linked and co-evolving traits of ethanol tolerance and yield. For example, improvement in both ethanol tolerance and yields were achieved by screening strain libraries overexpressing mutant genes in the eukaryote S. cerevisiae (Alper et al., 2006; Hong et al., 2010) or via genomic shuffling in Clostridium acetobutylicum (Mao et al., 2010). However, these approaches required a set of predetermined candidate genes (e.g., two TFs, spT15 and taf25 were selected as the targets to generate mutation libraries by gTME (Alper et al., 2006)) or laborious mutant selection steps (Alper et al., 2006; Hong et al., 2010; Mao et al., 2010), limiting them to a narrow range of hosts (e.g., well studied model organisms). In fact, simultaneous engineering of ethanol tolerance and ethanol titer has not been demonstrated in thermophilic bacteria, probably due to the scariness of prior biological knowledge. Unpublished work in our group suggested that productivity and tolerance of ethanol of the thermophiles can be simultaneously improved by either genetic (overexpressing several key regulatory and metabolic genes) or non-genetic approaches (e.g., medium supplementation of growth factors).

5. Genetic engineering in the thermophiles

5.1. Barriers for genetic manipulation of thermophilic bacteria

Despite the interest of employing the thermophiles in biofuel production (as described above; Fig. 1), rapid advances in strain development required well-established genetic tools. However, the thermophiles especially those low-G + C TGPAs were generally considered as more recalcitrant to genetic manipulation (Taylor et al., 2009), probably due to their unique genetic and physiological features. First, thermophilic bacteria cell envelope, formation of endospore and low permeability of plasma membrane might hinder efficient transformation of the cells (Averhoff, 2004; Culha et al., 2008; Silhavy et al., 2010). Second, thermostable replication origin of the transformed plasmid DNA is one important factor influencing plasmid stability in the thermophilic host. Third, thermostable and efficient marker-genes are also essential for genetic manipulation of the thermophiles, especially for thermophilic anaerobes. For example, the gfp gene, which is frequently used as a reporter gene in mesophilic aerobes, was not functional under thermoanaerobic conditions (Heim et al., 1994; Lin et al., 2010). Finally, reported thermostable transformation protocols are limited (Mai et al., 1997; Peng et al., 2006; Tyurin et al., 2004). Bacterial transformation techniques mainly include chemical transformation, conjugation, transduction and electroporation. However, chemical transformation, conjugation and transduction are fastidious in their host range. Conjugation requires a specific DNA donor to archive bacterial DNA transfer. However, such DNA donor cells were reported for only a few thermophiles (Cesar et al., 2011; Ramirez-Arcos et al., 1998; Wahlund and Madigan, 1995), thus limiting the application of conjugation on the genetic manipulation of those none naturally competent Gram-positive thermophilic bacteria. Although the host range suitable for electroporation might be wider, there are several drawbacks associated with this approach, such as the laboriousness of experimentation, the inaccessibility of most laboratories to customer-made pulse generators, the requirement of ion-free conditions and the generally low rate of cellular survival. These limitations have hindered broad application of the approach in the scientific community of the thermophiles, especially for those working on TGPAs (Table 1).

5.2. Development of genetic transformation and selection techniques

Despite these barriers, progresses have been made in genetic tools targeting the thermophiles during recent years (Cava et al., 2009; Lin et al., 2010; Suzuki and Yoshida, 2012). Electroporation-based transformation were reported for Thermoaerobacter (Lin et al., 2010; Peng et al., 2006), Moorella thermoacetica (Kita et al., 2012) and Thermoaerobacterium species (Mai et al., 1997; Shaw et al., 2008; Yao and Mikkelsen, 2010). High-efficiency DNA transformation was achieved for the usually calcitrant C. thermocellum, via electroporation with sophisticated customer-built cuvettes and pulse generators (Guss et al., 2012; Olson and Lynd, 2012b; Tyurin et al., 2004). In addition, natural genetic competence was reported in Thermus spp. (Cava et al., 2009; Koyama et al., 1986) as well as in thirteen Thermoaerobacter and Thermoaerobacterium strains (Shaw et al., 2010). Sonoporation exploits acoustic cavitation to create pores on cell membranes and thus delivers DNA and other macromolecules into the cell (Lin et al., 2010; Song et al., 2007). For Thermoaerobacter sp. XS14, we have developed an ultrasound-based sonoporation approach (Lin et al., 2010), which was validated by functionally expressing a foreign [-1,4-glucanase in vivo in the thermoanaerobic host. The transformation efficiency is six times higher than that in a parallelly performed yet slower and more tedious electroporation experiment (Lin et al., 2010). Additional studies have shown that sonoporation is applicable to many thermophilic bacteria of bioenergy relevance, including C. thermocellum, A. thermophilum and Caldicellulosiruptor BO47 (Yang and Li, 2010). Ultrasound-based sonoporation is particularly useful for transformation of thermophilic anaerobes, mainly due to its non-invasive and in-situ nature (Table 1).

After the delivery of foreign DNA, Restriction–Modification (R–M) systems of the host strains can become the next barrier for efficient transformation as it might digest “improperly” methylated incoming DNA and thus result in low transformation efficiency. Thermophilic bacteria appear to be no exception. To circumvent such barriers, a host-mimicking strategy was developed for Geobacter kasuithophilus HTA426 and Moorellathermoacetica ATCC39073 (Kita et al., 2012; Suzuki and Yoshida, 2012). In this strategy, DNA methyltransferases from these difficult-to-transform thermophilic strains were first introduced into an intermediate host such as E. coli; the intermediate host was then employed to produce plasmids with the “proper” methylation pattern, which thus enables the plasmids to overcome the R–M systems of the targeted thermophilic strains.

A number of thermostable plasmids designed for the genetic toolbox of the thermophiles have been reported, especially in Thermus spp. (Cava et al., 2009; Taylor et al., 2009; Tyurin et al., 2006), such as pNHK101, pTBA and pMK18 for Thermus thermophilus (Aoki and Itoh, 2007; Cava et al., 2009; Kobayashi et al., 2005), pDH10 for Thermotoga (Han et al., 2012), and temperature-sensitive (Ts) plasmids for TGPAs (Olson and Lynd, 2012a). These thermostable, high-copy number plasmids have enabled efficient overexpression of foreign/native genes in thermophilic hosts. In addition, the development of Ts plasmids, which at lower temperature replicate normally but at elevated temperature fail to replicate and thus become suicide vectors, has led to demonstrations that gene deletion via homologous recombination without high transformation efficiency is feasible for TGPAs (Olson and Lynd, 2012a).

Thermostable antibiotic-based selection systems have also been under rapid development. For example four thermostable antibiotic selection markers were reported in Thermus spp., such as the thermostable kanamycin, hygromycin and bleomycin resistant genes (Cava et al., 2007, 2009; Lasa et al., 1992). Moreover, functional selection systems at high temperature have been intensively studied. These systems select transformants based on functionally expressed proteins or the recovery from auxotrophy, and thus eliminate the false positive transformants. For example, a superfolder GFP (sGFP) that can functionally work at high temperature was reported (Pedelacq et al., 2006) and
has found wide applications in thermophilic bacteria, such as tracing intracellular locations of proteins in *T. thermophilus* at 70 °C (Cava et al., 2008), identifying secretory proteins in *T. thermophilus* (Cava et al., 2008), and the development of promoter probe plasmids in aerobic thermophiles (Cava et al., 2009). In addition, the pyf-based positive genetic system and hpt-based counter-selections system were reported in both *T. thermophilus* (Cava et al., 2009) and *C. thermocellum*, which produce marker-free mutants in *C. thermocellum* via complementary auxotroph (Argyros et al., 2011; Tripathi et al., 2010).

### 5.3. Development of genome manipulation techniques

Random insertion based on transposons (Pozsgai et al., 2012), directed insertion/deletion based on homologous recombination (Baba et al., 2006) or Group II introns (Heap et al., 2007), transduction (Lang and Beatty, 2000, 2002) and large-scale genome engineering such as DNA shuffling (Cohen, 2001) have been established for genome manipulation in several mesophiles. However, direct adaptation of these techniques to the thermophiles usually failed, probably due to heat inactivation of key components of these toolsets (Olson and Lynd, 2012a,b; Taylor et al., 2009).

So far in the thermophiles, a few more mature genetic techniques were mainly for the overexpression of foreign genes (which mainly requires stably replicating plasmids in thermophilic cells) and for directed gene knock-out mutagenesis via homologous recombination (which employs suicide vectors that are universal in bacteria) (Demain et al., 2005; Olson et al., 2010). In *T. ethanolicus* JW200, overexpression of native adhE via shuttle vector pTE16 improved ethanol production, while overexpression of heterologous cellulosomes in *Thermoanaerobacterium* (*T. saccharolyticum* JW/SY-5485 (Mai and Wiegel, 2000)) and *Thermoanaerobacterium* (*X514 via pK1M1 (Lin et al., 2010)) converted these thermoanerobic hosts into prototypic consolidated bioprocessing organisms that are not only ethanologenic but cellulolytic. In *C. thermocellum*, the pyf-based and hpt-based genetic selection tools for targeted gene deletion via homologous recombination enabled creating the Δpta and ΔptaΔadh mutants that nearly abolished acetate production (or both acetate and lactate production) and increased ethanol yield (Argyros et al., 2011; Tripathi et al., 2010). Progresses were also reported in additional thermophilic anaerobes of bioenergy relevance such as *Thermoanaerobacterium* and *Thermoanaerobacterium*, where branch fermentation pathways (e.g., lactate and acetate) were blocked and/or electron transfer systems were modified to enhance ethanol yields through gene deletion by homologous recombination (Table 2) (Chang and Yao, 2011; Demain et al., 2005; Shaw et al., 2008, 2009; Taylor et al., 2009; Yao and Mikkelsen, 2010). Technical details about gene overexpression and gene deletion in the thermophiles can be found at several excellent review articles (Olson and Lynd, 2012b; Taylor et al., 2009; Tyurin et al., 2006).

However, the requirement of high transformation efficiency (~10^5 CFU/μg DNA) has limited homologous recombination to only a few thermophiles. Thus, genome engineering strategies that do not require high transformation efficiency, such as ClosTron (based on Group II introns; (Heap et al., 2007)), random insertions via transposons (Pozsgai et al., 2012) and genetic exchange via bacteriophage (Lang and Beatty, 2000), might be promising for those thermophiles with lower transformation efficiency. Although no studies have yet reported their applications under high temperature, isolating such thermostable genetic elements in the thermophiles might be feasible. For example, recent analysis of native insertion sequences (simple transposable elements that can also be parts of composite transposons such as Tn5 (Mahillon and Chandler, 1998)) in thermophilic cyanobacteria could serve as the foundation for the development of transposon-based random insertion in the thermophiles (Nelson et al., 2011). Moreover, the recently isolated *Thermus*-specific phages (Cava et al., 2009; Sevostyanova et al., 2007) might potentially serve as gene-transfer agents for phages-mediated transduction in the thermophiles.

### 6. Conclusions and future perspectives

With the unique advantages in biofuel production and the progress in systems-level understanding and engineering (as reviewed here), several thermophilic bacteria (such as *T. saccharolyticum* JW/SY-5485, *C. thermocellum* DSM1313 and *Thermoanaerobacterium* sp. X514) are emerging as research models of functional genomics for carbon catabolism (Lin et al., 2011; Vanfossen et al., 2009), stress response and adaptation (Brown et al., 2011; Taylor et al., 2009), and metabolic engineering (Argyros et al., 2011; Shaw et al., 2008; Yao and Mikkelsen, 2010). Moreover, genome sequences of novel thermophiles with exceptional genetic or physiological features are emerging at an unprecedented rate (Elkins et al., 2006).

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Table 1

<table>
<thead>
<tr>
<th>Technique</th>
<th>In situ, non-cell-contacting and non-invasive; genetic transformation of cells in their native growth media</th>
<th>Highly scalable. It can be applied to large bioreactors and natural environments</th>
<th>Gram-positive and Gram-negative bacteria; potentially applicable to archaea</th>
<th>Easy</th>
<th>Possible</th>
<th>Simple and rapid; easy to adapt to high-throughput; particularly advantageous for strictly anaerobic bacteria</th>
<th>Low cost for device and no consumables</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scalability</strong></td>
<td>In situ</td>
<td>Difficult to scale up as chemicals were introduced that might be difficult to contain, recover or recycle</td>
<td>Mostly Proteobacteria and Euryarchaeota</td>
<td>Mostly plant cells</td>
<td>Mostly plant cells</td>
<td>Most tedious and cumbersome; requiring pretreatment of host cells</td>
<td>Low cost for device and consumables</td>
</tr>
<tr>
<td><strong>Invasiveness to the cell</strong></td>
<td>Maximal cellular viability due to the quick self-repair of the cell membranes</td>
<td>Maximal cell viability due to the quick self-repair of the cell membranes</td>
<td>Cell survival can be drastically reduced due to damage to the cell membrane</td>
<td>Wide host-range; mostly for bacteria and archaea</td>
<td>Wide host-range; mostly for bacteria and archaea</td>
<td>More tedious and cumbersome; adding glycine or cell wall-weakening agents; laborious pretreatment, e.g. chilling and repetitious washing anaerobically; more difficult to achieve high-throughput</td>
<td>Costly device and consumables</td>
</tr>
<tr>
<td><strong>Host range</strong></td>
<td>Wide host-range that includes both Gram-positive and Gram-negative bacteria</td>
<td>Mostly Proteobacteria and Euryarchaeota</td>
<td>Mostly plant cells</td>
<td>Wide host-range; mostly for bacteria and archaea</td>
<td>Wide host-range; mostly for bacteria and archaea</td>
<td>More difficult</td>
<td>Costly device and consumables</td>
</tr>
<tr>
<td><strong>Remote-control and automation</strong></td>
<td>Ex situ</td>
<td>Ex situ; addition of cations or chemical reagents</td>
<td>Ex situ; delivery of DNA into the cell via a specialized biolistic particle delivery system</td>
<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
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<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
</tr>
<tr>
<td><strong>Simplicity and throughput</strong></td>
<td>Ex situ</td>
<td>Ex situ; addition of cations or chemical reagents</td>
<td>Ex situ; delivery of DNA into the cell via a specialized biolistic particle delivery system</td>
<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
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<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
</tr>
<tr>
<td><strong>Costs</strong></td>
<td>Ex situ</td>
<td>Ex situ; addition of cations or chemical reagents</td>
<td>Ex situ; delivery of DNA into the cell via a specialized biolistic particle delivery system</td>
<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
<td>Ex situ transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box</td>
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et al., 2010; Nelson et al., 2011), laying a broad and solid foundation for system-level understanding and engineering (Table 2). These efforts will undoubtedly expand from the more conventional population-level analysis into both the single-cell level and the consortia level analyses, where the genetic nature and phenotypic heterogeneity of industrial analysis into both the single-cell level and the consortia level analyses, will undoubtfully expand from the more conventional population-level analysis into both the single-cell level and the consortia level analyses, laying a broad and solid foundation for Current strategies for genetic engineering of model thermophilic bacteria. 

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Acknowledgment

Table 2

<table>
<thead>
<tr>
<th>Target traits</th>
<th>Hosts</th>
<th>Strategy</th>
<th>Results</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Improved ethanol titer</td>
<td>Thermoanaerobacter saccharolyticum JW/SL-Y5485</td>
<td>Genetic strategy (a “carbon centered” approach): knockout of genes involved in acetate and lactate formation</td>
<td>● Improved ethanol yields (37 g/L at mixed sugars in 24 h batch fermentation)</td>
<td>Shaw et al., 2008</td>
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<tr>
<td></td>
<td></td>
<td>Genetic strategy (an “electron centered” approach): knockout of ferredoxin-linked hydrogenate and lactate dehydrogenase genes</td>
<td>● Lower cell yield (20% lower)</td>
<td>Shaw et al., 2009</td>
</tr>
<tr>
<td>Clostridium thermocellum DSM1313</td>
<td></td>
<td>Genetic strategy: knockout of psa involved in acetate formation</td>
<td>● Improved ethanol yields (0.39 g per gram consumed glucose equivalent in 24 h batch fermentation)</td>
<td>Georgieva et al., 2008</td>
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<td></td>
<td>Geobacillus thermodenitrificans</td>
<td></td>
<td>● Growth defect (50% lower cell density)</td>
<td></td>
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<tr>
<td>Improved ethanol titer</td>
<td>Thermoanaerobacter ethanolicus 39E, C. thermocellum LQRI</td>
<td>Non-genetic strategy: co-culture and medium supplementation of vitamin B12</td>
<td>● Improved ethanol yields (40 mM (with B12) vs 10 mM (without B12))</td>
<td>Hemme et al., 2011</td>
</tr>
<tr>
<td>Improved ethanol titer</td>
<td>Thermoanaerobacter saccharolyticum JW/SL-Y5485</td>
<td>Genetic engineering, fermentation improvement and adaptive evolution: gene deletion, co-culture and sequential transfers</td>
<td>● Increased cost</td>
<td>Argyros et al., 2011</td>
</tr>
<tr>
<td>Improved ethanol tolerance</td>
<td>Thermoanaerobacter ethanolicus 39E</td>
<td>Chemical mutagenesis</td>
<td>● Faster growth</td>
<td></td>
</tr>
<tr>
<td>Improved ethanol tolerance</td>
<td>Clostridium thermocellum ATCC 27405</td>
<td>Sequential transfers</td>
<td>● 8% (v/v) ethanol tolerance</td>
<td>Burdette et al., 2002</td>
</tr>
<tr>
<td>High biofuel yields, robustness and cell yield</td>
<td>The thermophiles</td>
<td>Genetic and non-generic engineering based on system-level understanding</td>
<td>● A level of lactate production three times of that in WT</td>
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<td></td>
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<td></td>
<td>● 80% reduction of ethanol consumption</td>
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<td></td>
<td></td>
<td></td>
<td>● Less ethanol yields</td>
<td>Brown et al., 2011; Timmons et al., 2009</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>● Higher biofuel yields</td>
<td>See a perspective in Fig. 2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>● Higher tolerance to the end products (e.g. biofuels)</td>
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<td></td>
<td></td>
<td></td>
<td>● Higher robustness of production in industrial conditions</td>
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</table>


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et al., 2010; Nelson et al., 2011), laying a broad and solid foundation for system-level understanding and engineering (Table 2). These efforts will undoubtedly expand from the more conventional population-level analysis into both the single-cell level and the consortia level analyses, where the genetic nature and phenotypic heterogeneity of industrial relevant traits (e.g. Carlquist et al., 2012) in the thermophiles and the thermophilic bioprocesses are interpreted and engineered via high-throughput omics and non-invasive phenotyping technologies at the single-cell resolution (Fernandes et al., 2011; Kalisky et al., 2011; Li et al., 2012). Finally, technological development in large-scale engineering and synthesis technology of thermophilic genomes and cellular networks, although still at the nascent stage, promises to substantiate a synthetic biology perspective, where functional parts, module, chassis, cells and consortia from thermophilic microbes are designed, assembled and employed for specialized missions in natural or industrial environments.

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