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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 osmic 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.

Contents

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

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thermophilic (Cava et al., 2009; Wiegel and Adams, 2003). Furthermore, these extraordinary life-forms have found extensive applications in bioindustry (Blumer-Schuette 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 *Thermoanaerobacter* 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 gyrases, 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

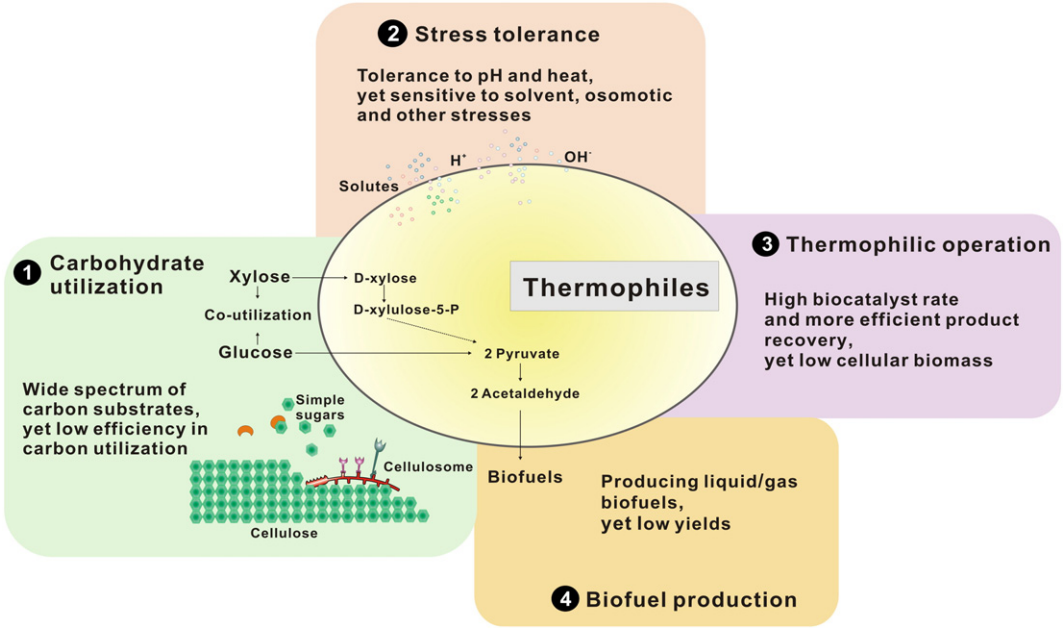

Fig. 1. *Advantages and disadvantages of the thermophiles for the industrial productions of fuels and chemicals.* C5: pentose; C6: hexose; S: solvents.

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 carbohydrates, 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 polysaccharides 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 hyperthermophilic marine bacteria ($T_{opt} \geq 80$ °C, *e.g.*, *Thermotogales*) can catabolize 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 thermopiles, those with upper growth-temperature limit near 78 °C frequently degrade cellulose *via* "free-acting" cellulases, as represented by the two genera *Anaerocellum* (*Anaerocellum thermophilum*, T_{opt} 75 °C) and *Caldicellulosiruptor* (*Caldicellulosiruptor kristjanssonii*, T_{opt} 78 °C; *Caldicellulosiruptor bescii*, T_{opt} 80 °C) (Blumer-Schuette et al., 2008; Dam 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 *Caldicellulosiruptor* 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 *Caldicellulosiruptor* (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 (Dam 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-modular, multifunctional 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 cellulolytic enzyme complexes and consist of nonenzymatic scaffolding proteins and cellulases, which are produced by cellulolytic mesophilic and thermophilic anaerobes 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 regulation 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 cellulolytic *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. Hexose and pentose co-utilization is a highly valuable trait among biofuel-producing microbes. However, most known mesophilic cellulose degrader or ethanologens 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 hexose 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 absolute carbon flux from xylose and thus likely increases ethanol production (Hemme et al., 2011). Moreover, genome-wide regulatory networks of the "thermophilic" glycobiome 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 glycobiome, 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* glycobiome along the bacterial growth course revealed the cooperating nature of pentose- and hexose-catabolism in this organism. Specifically, glucose accelerates xylose utilization *via* activating xylose 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 regulatory 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 O_L and O_R to block

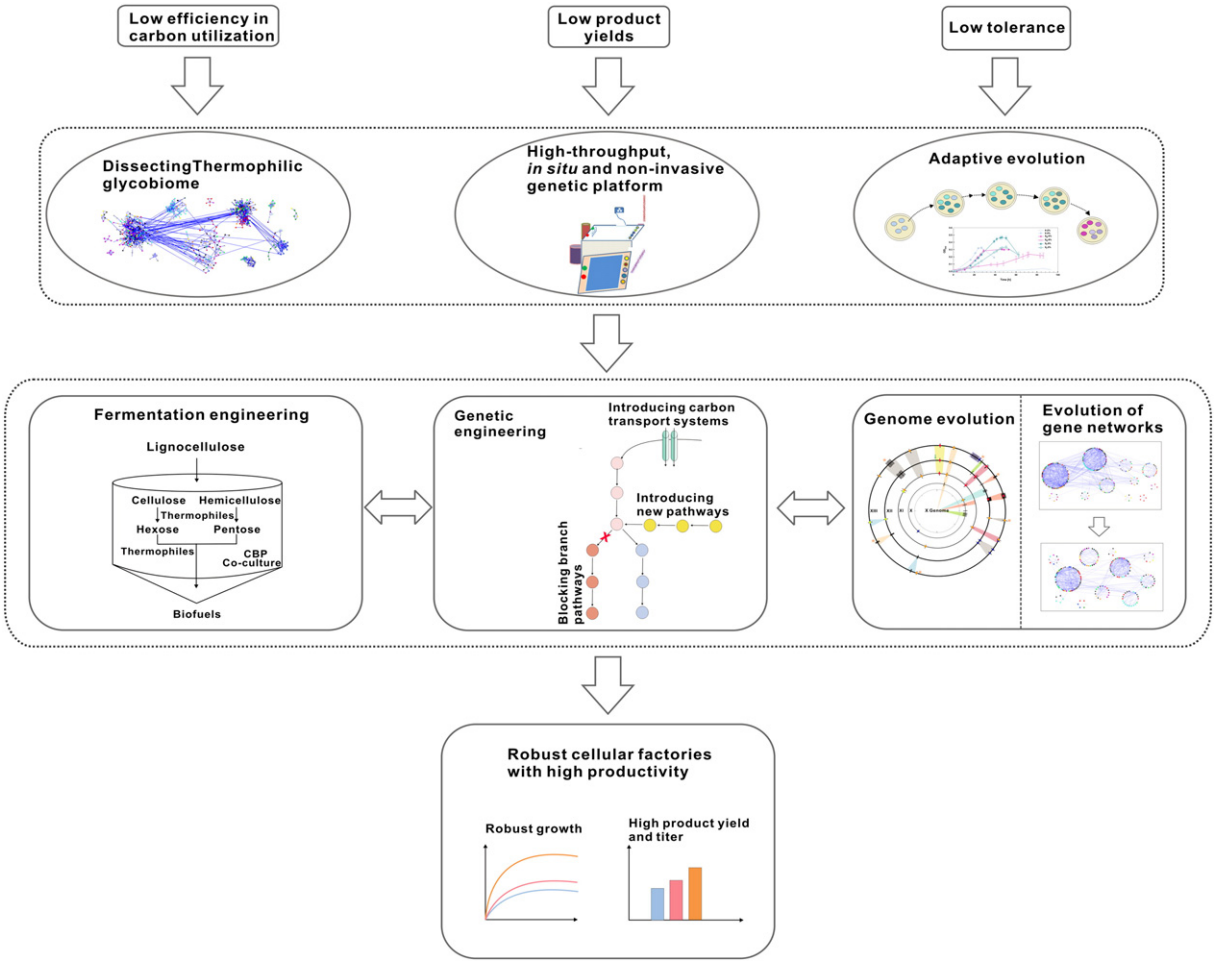

Fig. 2. Roadmap of dissecting and engineering thermophilic bacteria for industrial production of fuels and chemicals.

xyl-loci 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 *xyl*A), 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, σ^{32}, 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*

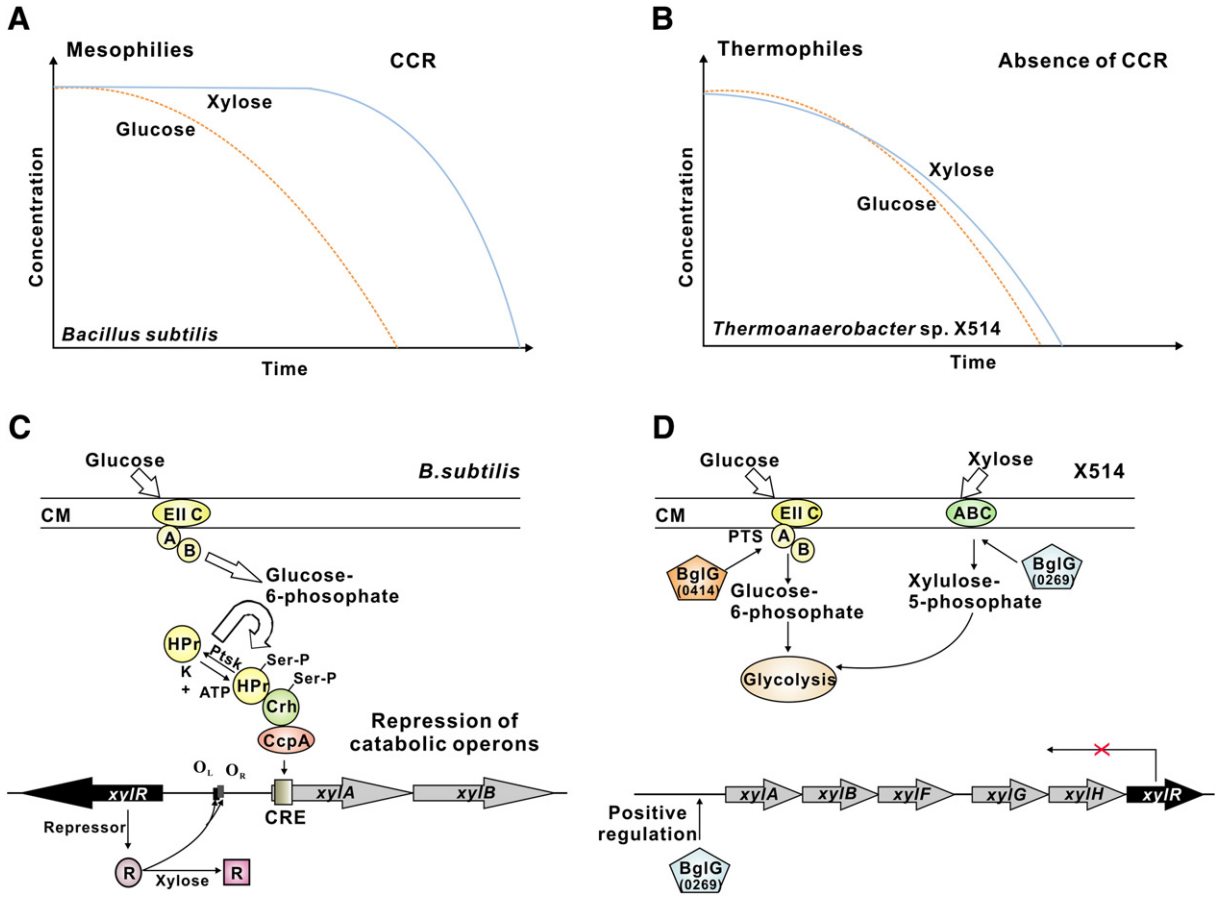

Fig. 3. *Molecular mechanisms for the co-utilization of pentose and hexose in certain thermophilic bacteria.* Fermentation of glucose and xylose in a mesophilic bacteria *Bacillus subtilis* (A) and in a thermophilic bacteria *Thermoanaerobacter* sp. X514 (B). (C) Regulation of the carbon utilization that prefers glucose in *Bacillus subtilis*. XylR, as a repressor, binds to the xyl operators to inhibit gene expressions in *xyl* loci. When xylose is present, this repression is derepressed *via* the binding of xylose to XylR which changes the protein confirmation. Under glucose or under glucose plus xylose, the activated Hpr interacts with CcpA to bind CRE, resulting in the CCR (Dahl and Hillen, 1995). Ptsk: HPr kinase. (D) Regulation of glucose and xylose co-utilization in *Thermoanaerobacter* sp. X514. The *Thermoanaerobacter* glycobiome suggested that both glucose and xylose-transport systems are apparently both regulated by BglGs, rather than by XylR for pentose.

(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., *Desulforudis 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_{opt} of the organism. For instance, in the mesophiles, an increase in the influx of K^+ ion is the earliest response to osmotic upshock, and then major anionic compounds (glutamate) are synthesized and accumulated; after the decline of K^+/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

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 *Clostridum thermohydrosulfuricum*, 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 (soxS, marA and robA encoding DNA-binding proteins/transcriptional activators), mannose transporter of the phosphotransferase system (*man*XYZ), 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) (Burdette et al., 2002). In *C. thermocellum*, a mutated *adh*E 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; Wiegel 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 discrepant environments (Barrick et al., 2009; Bromham, 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, 2009). Drake explained this phenomenon by organismal adaption to avoid deleterious mutations at high temperature (Drake, 2009). 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 scarceness 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 thermophilic 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 *Thermoanaerobacter* (Lin et al., 2010; Peng et al., 2006), *Moorella thermoacetica* (Kita et al., 2012) and *Thermoanaerobacterium* species (Mai et al., 1997; Shaw et al., 2008; Yao and Mikkelsen, 2010). High-efficiency DNA transformation was achieved for the usually recalcitrant *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 *Thermoanaerobacter* and *Thermoanaerobacterium* 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 *Thermoanaerobacter* sp. X514, we have developed an ultrasound-based sonoporation approach (Lin et al., 2010), which was validated by functionally expressing a foreign β-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* OB47 (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 *Geobacillus kaustophilus* 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, pTT8 and pMK18 for *Thermus thermophiles* (Aoki and Itoh, 2007; Cava et al., 2009; Kobayashi et al., 2005), pDH10 for *Thermotaga* (Han et al., 2012), and temperature-sensitive (T_S) 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 T_S 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

Table 1
Comparison between sonoporation and other approaches for the transformation of thermophilic bacteria.

	Sonoporation (Lin et al., 2010)	Chemical transformation (Aune and Aachmann, 2010)	Gene gun bombardment (Gan et al., 2000)	Electroporation (Olson and Lynd, 2012b)
In situ	*In situ*, non-cell-contacting and non-invasive: genetic transformation of cells in their native growth media	*Ex situ*; adding cations or chemical reagents	*Ex situ*; delivery of DNA into the cell *via* a specialized biolistic particle delivery system	*Ex situ* transformation in low-ionic strength buffer housed in specialized cuvettes; all in a anaerobic glove box
Scalability	Highly scalable. It can be applied to large bioreactors and natural environments	Difficult to scale up as chemicals were introduced that might be difficult to contain, recover or recycle	Non-scalable	Non-scalable
Invasiveness to the cell	Maximal cell viability due to the quick self-repair of the cell membranes	Maximal cell viability due to the quick self-repair of the cell membranes	Cell survival can be drastically reduced due to damage to the cell membrane	Cell survival can be drastically reduced due to damage to the cell membrane
Host range	Wide host-range that includes both Gram-positive and Gram-negative bacteria; potentially applicable to all bacteria and archaea	Mostly Proteobacteria and Euryarchaeota	Mostly plant cells	Wide host-range; mostly for bacteria and archaea
Remote-control and automation	Easy	Possible	More difficult	More difficult
Simplicity and throughput	Simple and rapid; easy to adapt to high-throughput; particularly advantageous for strictly anaerobic bacteria	Simple and rapid, but usually requiring preparation of competent cell and manipulation at low temperature	More tedious and cumbersome: requiring pretreatment of host cells	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
Costs	Low cost for device and no consumables	Low cost for device and consumables	Costly device and consumables	Costly device and consumables

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 *adh*E *via* shuttle vector pTE16 improved ethanol production, while overexpression of heterologous cellulases in *Thermoanaerobacterium* (*T. saccharolyticum* JW/SL-YS485 (Mai and Wiegel, 2000)) and *Thermoanaerobacter* (X514 *via* pIKM1 (Lin et al., 2010)) converted these thermoanaerobic hosts into prototypic consolidated bioprocessing organisms that are not only ethanologenic but cellulolytic. In *C. thermocellum*, the *pryF*-based and *hpt*-based genetic selection tools for targeted gene deletion *via* homologous recombination enabled creating the Δ*pta* and Δ*pta*Δ*ldh* 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 *Thermoanaerobacter*, 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/SL-YS485, *C. thermocellum* DSM1313 and *Thermoanaerobacter* 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

Table 2
Current strategies for genetic engineering of model thermophilic bacteria.

Target traits	Hosts	Strategy	Results	References
Improved ethanol titer	*Thermoanaerobacterium saccharolyticum* JW/SL-YS485	Genetic strategy (a "carbon centered" approach): knockout of genes involved in acetate and lactate formation	● Improved ethanol yields (37 g/L at mixed sugars in 24 h batch fermentation) ● Lower cell yield (20% lower)	Shaw et al., 2008
		Genetic strategy (an "electron centered" approach): knockout of ferredoxin-linked hydrogenate and lactate dehydrogenase genes	● Improved ethanol yields (0.35 g per gram consumed glucose equivalent in 24 h batch fermentation) ● Growth defect (50% lower cell density)	Shaw et al., 2009
	Thermoanaerobacter mathrani BG1L1	Genetic strategy: knockout of *pta* involved in acetate formation	● Improved ethanol yields (0.39–0.42 g per gram sugars consumed with wheat straw hydrolysate in a 143-day continuous fermentation)	Georgieva et al., 2008
	Clostridium thermocellum DSM1313	Genetic strategy: knockout of *pta* involved in acetate formation	● Improved ethanol yields (~0.8 g/L ethanol at 0.5 g/L cellobiose in 48 h batch fermentation) ● Lower growth rate (1/3 lower) ● One-third less biomass than WT	Tripathi et al., 2010
	Geobacillus thermoglucosidasius	Genetic strategy: knockout of *pfl* and *ldh* and upregulation of a *pdh*	● Improved ethanol yields (~0.42 g/g ethanol on glucose) ● A slower metabolism on xylose than hexoses	Cripps et al., 2009
Improved ethanol titer	*Thermoanaerobacter ethanolicus* 39E, *C. thermocellum* LQRI	Non-genetic strategy: co-culture and medium supplementation of vitamin B_{12}	● Improved ethanol yields (40 mM (with B_{12}) vs 10 mM (without B_{12})) ● Increased cost	Hemme et al., 2011
Improved ethanol titer	*C. thermocellum* DSM1313 *Thermoanaerobacterium saccharolyticum* JW/SL-YS485	Genetic engineering, fermentation improvement and adaptive evolution: gene deletion, co-culture and sequential transfers	● Improved ethanol titer (38.1 g/L ethanol at 92.3 g/L Avicel for 146 h, batch co-culture fermentation) ● Faster growth	Argyros et al., 2011
Improved ethanol tolerance	*Thermoanaerobacter ethanolicus* 39E	Chemical mutagenesis	● 8% (v/v) ethanol tolerance ● A level of lactate production three times of that in WT ● 80% reduction of ethanol consumption	Burdette et al., 2002
Improved ethanol tolerance	*Clostridium thermocellum* ATCC 27405	Sequential transfers	● 8% (v/v) ethanol tolerance ● Less robustness (40% lower cell yield) ● Less ethanol yields	Brown et al., 2011; Timmons et al., 2009
High biofuel yields, robustness and cell yield	The thermophiles	Genetic and non-genetic engineering based on system-level understanding	● Higher biofuel yields ● Higher tolerance to the end products (*e.g.* biofuels) ● Higher robustness of production in industrial conditions	See a perspective in Fig. 2

et al., 2010; Nelson et al., 2011), laying a broad and solid foundation for system-level understanding and engineering (Table 2). These efforts will undoubtfully 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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