

Minireview

Genetic tool development underpins recent advances in thermophilic whole-cell biocatalysts

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Summary

The environmental value of sustainably producing bioproducts from biomass is now widely appreciated, with a primary target being the economic production of fuels such as bioethanol from lignocellulose. The application of thermophilic prokaryotes is a rapidly developing niche in this field, driven by their known catabolic versatility with lignocellulose-derived carbohydrates. Fundamental to the success of this work has been the development of reliable genetic and molecular systems. These technical tools are now available to assist in the development of other (hyper)thermophilic strains with diverse phenotypes such as hemicellulolytic and cellulolytic properties, branched chain alcohol production and other 'valuable bioproduct' synthetic capabilities. Here we present an insight into the historical limitations, recent developments and current status of a number of genetic systems for thermophiles. We also highlight the value of reliable genetic methods for increasing our knowledge of thermophile physiology. We argue that the development of robust genetic systems is paramount in the evolution of future thermophilic based bioprocesses and make suggestions for future approaches and genetic targets that will facilitate this process.

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Thermophiles and their bioproducts

Scientific interest in thermophiles can be divided into three main endeavours: (i) the general isolation, characterization and exploration of thermophilic life and the boundaries defining its limits, (ii) the physiological and biochemical characterization of the various adaptive mechanisms required for microbial survival at high temperatures, and (iii) the characterization and development of thermostable biocatalysts/bioproducts. Advances in the first two areas have been well summarized elsewhere (Gerday and Glansdorff, 2007; Robb *et al.*, 2008) but developments in thermophilic whole-cell biocatalysts have, with a few exceptions, been modest.

Although many enzymes from thermophilic organisms have reached full commercialization (the best-known examples being a number of polymerases such as *Taq* and *Pfu*) the general commercial approach to the bulk production of commercial thermostable enzymes has been to engineer thermostability rather than seek it from a thermophilic organism/source material (Haki and Rakshit, 2003). This approach is perfectly sound and perhaps has arisen because of the greater mesophilic diversity in genomic databases. It has resulted in a wide range of thermostable enzyme mutants that are applicable across a broad range of biotechnological targeted markets such as the food, feed and textile industries (Turner *et al.*, 2007).

The industrial use of thermophilic whole-cell biocatalysts has been widely anticipated, but has largely remained undeveloped. Some advantages in the use of thermophiles as whole-cell biocatalysts are: (i) for anaerobic strains, high-temperature fermentations retain anaerobic status more readily, (ii) thermophiles may have lower sensitivity to organic solvents, (iii) there may be a reduced risk of contamination and (iv) the ability to operate at elevated temperatures allows the chemistry of some processes to be 'accelerated' (Zeikus *et al.*, 1981). Drawbacks include the technical challenges of high-temperature culturing and differences in codon usage and folding processes which may lead to low levels of expression or recombinant enzymes with reduced or no activity (Haki and Rakshit, 2003; Turner *et al.*, 2007). Process economics may also be exacerbated through associated heating costs.

A major barrier to development of thermophilic biocatalysts has been the general inability to genetically modify the parent strains. Until recently there has been a dearth of reliable methods for inducing competence, genetic material transfer, gene expression and genome integration in those thermophilic genera that have been identified as being of biotechnological value. The most recent examples of strain development success have ultimately derived from genetic systems development.

Thermostable genetic systems: overcoming the barriers to development

Recent genetic method development programmes have been very successful in developing a range of commercially relevant organisms. While the strategies used have similar themes, it is informative to review the issues that have historically restricted genetic system advancement in thermophilic bacteria. Although the recent and tangible advances have been made with thermophilic prokaryotes, for the sake of completeness, the hyperthermophilic archaea will also be discussed, since the burden of working at high temperatures is entirely transferable across all (hyper)thermophiles. The reader is directed to several recent and well-written reviews for a more complete understanding of thermophilic archeal genetic tools available (Berkner and Lipps, 2008; Wang *et al.*, 2009). Examples are included in Tables 1 and 2 for convenience.

Transformation

The ability to introduce and then recover/identify genetic material from a host strain is crucial to the establishment of a molecular toolkit. For thermophiles, natural competency appears rare and where it does exist, low transformation efficiencies are typical, either because DNA uptake systems simply are not present or because the conditions required for their activity have not been met (Cava *et al.*, 2009). As a result, early competency methods centred on the generation of protoplasts [e.g. *Geobacillus* spp. (Wu and Welker, 1989)] with subsequent cellular regeneration.

The description of cell membrane permeabilization by the application of electric fields (electroporation) in 1982 and the availability of commercial electroporators in the mid-1980s revolutionized transformation methods generally and led to the development of efficient, rapid and reproducible transformation protocols for a variety of thermophilic genera. Such protocols commonly include high field strengths and exponential decay electroporation after prior treatment of cultures with cell wall-destabilizing compounds, which appears to generally improve efficiency (Klapatch *et al.*, 1996; Mai *et al.*, 1997; de Grado *et al.*, 1999; Iwai *et al.*, 2004; Tyurin *et al.*, 2004; Bjorns-

dottir *et al.*, 2005). There have been some notable successes, but significant intra-genus variability is evident, which needs explaining (Studholme *et al.*, 1999). Although electroporation is the apparent method of choice (Table 1), alternative transformation methods such as natural competency, protoplast transformation, chemical treatment with CaCl₂ and even heat shock (Wu and Welker, 1989; Noll and Vargas, 1997; de Grado *et al.*, 1999; Sato *et al.*, 2003; Cava *et al.*, 2009; Shaw *et al.*, 2010) have all been applied to thermophilic prokaryotic and archaeal spp. Successful transformation methods do not therefore, differ significantly between mesophiles and thermophiles in terms of the basic methodology, although there are no general rules emerging as yet. For the newcomer, the simplicity of electroporation as a procedure would tend to make it the first option and, where it works, higher efficiencies are generally achieved than with other methods. As transformation is often simply a means to an end, significant focused research into the mechanisms of natural competency may have suffered *in lieu* of achieving the ultimate goal, i.e. gene knockout or expression. Nevertheless, the identification of natural competency in *Thermus* spp. is resulting in significant understanding of the species at a genomic and molecular level (Friedrich *et al.*, 2001; 2002; 2003; Rumszauer *et al.*, 2006) and has even been used to compare to and further understand mesophilic pathogen/host tissue interactions (Friedrich *et al.*, 2001; 2002; 2003; Rumszauer *et al.*, 2006). In addition the application of the simple yet effective method of chemical treatment (CaCl₂) has proven effective among the thermophilic archaea again highlighting the suspicion that fundamentally, there may be little difference in achieving competency in thermophiles as compared with mesophiles (Sato *et al.*, 2003; Waeger *et al.*, 2010).

Vectors of genomic material

The most common vectors for genetic material transfer are plasmids, small self-replicating DNA units carrying independent replicons and markers for their selection and identification in host cells. Following the first reports of plasmids from thermophilic prokaryotes (Matsumura and Aiba, 1985; Koyama *et al.*, 1986) the cloning of undefined DNA fragments into standard *Escherichia coli* vectors and selection in a thermophile enabled identification of regions containing thermophilic origins of replication, forming the first 'shuttle vectors'. However, these did not have the versatility expected of modern vectors, which has required the generation of new families of more functional plasmids (Table 2).

A number of characteristics are desirable in a thermophilic genetic vector. These include a suitable origin of replication for plasmid maintenance in the host(s) at high temperatures and a suitable thermostable antibiotic

Table 1. Methods of competency and transformation for selected thermophilic prokaryotes of commercial relevance.

Organism	Genome sequence	Competency method	Transformation method	Maximum reported efficiency	Reference
<i>Thermoanaerobacterium saccharolyticum</i> JW/SL-YS485	Department of Energy (DOE) Joint Genome Institute	Autoplast generation with cell wall-destabilizing agent (niacin), subsequent sucrose-containing buffer washes	Electroporation (12.5 kV cm ⁻¹ , 400 ohm, 25 µF)	Approximately 10 ³⁻⁴	Tyurin et al. (2006), Shaw et al. (2008)
<i>Thermoanaerobacter ethanolicus</i> JW200	DOE Joint Genome Institute	Glycine- and sucrose-induced protoplast formation, subsequent glycerol-containing buffer washes	Electroporation (13 kV cm ⁻¹ , 400 ohm, 25 µF)	Approximately 10 ¹	Peng et al. (2006)
<i>Thermoanaerobacter mathranii</i> BG1	Not recorded	Cellobiose wash buffer. Isoniacin addition	Custom built electroporator with custom cuvettes (2mm gap). 10 ms square wave pulse. 25 kV cm ⁻¹ , 600 ohm, 10 µF	10 ²⁻⁵	Yao and Mikkelsen (2010)
<i>Geobacillus thermoglucosidasius</i> NCIMB 11955	Not recorded	High osmolarity washing buffer (sorbitol and mannitol)	Electroporation (25 kV cm ⁻¹ , 600 ohm, 10 µF)	10 ⁴	Taylor et al. (2008), Cripps et al. (2009)
<i>Geobacillus thermoglucosidasius</i> DL33	Not recorded	High osmolarity washing buffer (sorbitol and mannitol)	Electroporation (25 kV cm ⁻¹ , 600 ohm, 10 µF)	10 ³⁻⁴	Taylor et al. (2008), Cripps et al. (2009)
<i>Clostridium thermocellum</i>	DOE Joint Genome Institute	Isoniacin addition	Custom built electroporator with custom cuvettes (2mm gap). 10 ms square wave pulse. 25 kV cm ⁻¹	10 ³	Tyurin et al. (2004)
<i>Rhodothermus marinus</i> (tpB)	DOE Joint Genome Institute	Glycerol treatment	Electroporation (22.5 kV cm ⁻¹ , 200 ohm, 25 µF)	10 ⁶	Bjornsdottir et al. (2005; 2006; 2007)
<i>Thermosynechococcus elongatus</i> BP-1	Kasza DNA Research Institute	2mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) treatment	Electroporation (10 kV cm ⁻¹ , 200 ohm, 25 µF) and natural competency	10 ⁸	Iwai et al. (2004)
<i>Thermotoga maritima</i> , T. neapolitana	J. Craig Venter Institute	Lysozyme treatment and DNA encapsulation with liposome reagent	Spheroplast creation and cationic liposome integration	10 ²	Noll and Vargas (1997), Yu et al. (2001), Conners et al. (2006)
<i>Thermus thermophilus</i> HB27	Goettingen Genomics Laboratory	Glycerol treatment	Natural competency and electroporation (25 kV cm ⁻¹)	10 ⁹	Friedrich et al. (2001)
<i>Pyrococcus furiosus</i> DSM 3638	Universities of Utah and Maryland	CaCl ₂ treatment	Heat shock at 80°C	10 ²	Waage et al. (2010)
<i>Thermococcus kodakaraensis</i> KOD1	Kyoto University	CaCl ₂ treatment	Heat shock at 85°C	10 ²	Fukui et al. (2005), Sato et al. (2005)
<i>Sulfolobus islandicus</i> , S. solfataricus	University of Copenhagen, DOE Joint Genome Institute	Sucrose treatment in growth medium and wash buffers	Electroporation (7.5 kV cm ⁻¹ , 400 ohm, 25 µF)	10 ³	She et al. (2001; 2009), Albers and Driessen (2008)

Table 2. A summary of available vectors for selective thermophilic species.

Organism	Vectors (marker and origin)	Markers	Origins of replication	Genetic use	Reference
<i>Thermoanaerobacterium saccharolyticum</i> JW/SLYS485	pKM1 pSGD- series pHK- series pRKM1 pRUKM pTE16 pTEA2	Kan from pKD102 and Amp from pMLS Kan from pKM1 and Amp from pUC variant Kan from pKM1 and Ery from pSGD8-erm Kan from pKD102 and Cat from pRP9 Markers from pRKM1 and Amp from pUC variant Cat from pC194 and Ery from pJIR751 Cat from pC194 and Amp from pUC variant	<i>E. coli</i> and <i>Clostridium acetobutylicum</i> <i>E. coli</i> <i>E. coli</i> <i>E. coli</i> and <i>Bacillus</i> sp. <i>E. coli</i> and <i>Bacillus coagulans</i> <i>E. coli</i> and <i>Clostridium perfringens</i> <i>E. coli</i>	Shuttle vector, expression vector Suicide vector Suicide vector Shuttle vector Shuttle vector Shuttle vector Suicide vector	Mai <i>et al.</i> (1997), Tyurin <i>et al.</i> (2004) Desai <i>et al.</i> (2004) Shaw <i>et al.</i> (2008) Mai and Wiegel (2000) Mai and Wiegel (2000) Peng <i>et al.</i> (2006) Peng <i>et al.</i> (2006)
<i>Thermoanaerobacter ethanolicus</i> JW200	p3CHPT and derivatives pUB110 derivatives (pTMO-series)	Kan (unreported origin) and Amp from pUC variant Kan from pUB110 and Amp from pUC variant	<i>E. coli</i> <i>E. coli</i> and <i>Staphylococcus aureus</i>	Suicide and expression vector Integration vector	Yao and Mikkelsen (2010) Cripps <i>et al.</i> (2009)
<i>Geobacillus thermoglucosidasius</i> DL33	pUCG18	Kan from pBST22 and Amp from pUC variant	<i>E. coli</i> and <i>Geobacillus stearothermophilus</i>	Expression vector	Taylor <i>et al.</i> (2008)
<i>Rhodothermus marinus</i> (trpB)	pRM100 and pRM- series	Amp from pUC and native <i>trpB</i> gene	<i>E. coli</i> and <i>R. marinus</i>	Shuttle vector	Bjomsdottir <i>et al.</i> (2005)
<i>Thermosynechococcus elongatus</i> BP-1	RSF1010-derived plasmids	–	<i>E. coli</i> and <i>T. elongatus</i>	Shuttle vector	Muhlenhoff and Chauvat (1996)
<i>Thermotoga maritima</i> , <i>T. neapolitana</i>	pJY1, pJY2	Cat from pC194, Kan from pPP442 and Amp from pBluescript	<i>E. coli</i> and <i>T. maritima</i>	Shuttle vector	Yu <i>et al.</i> (2001)
<i>Thermus thermophilus</i> HB27	pMK- series	Kan from pEM2 and Amp from pUC variant	<i>E. coli</i> and <i>Thermus</i> sp.	Shuttle vector, expression vectors	de Grado <i>et al.</i> (1999), Moreno <i>et al.</i> (2003)
<i>Thermus thermophilus</i> pyrE- <i>Pyrococcus furiosus</i> DSM 3638	pINV pYS- series (pGT5 derived)	<i>pyrE</i> marker recovery <i>pyrF</i> marker and simvastatin resistance in subsequent plasmid improvements	<i>E. coli</i> <i>E. coli</i> and <i>Pyrococcus</i> origin from pGT5	Suicide/integration vector Shuttle vector	Tamakoshi <i>et al.</i> (1997) Erauso <i>et al.</i> (1996), Lucas <i>et al.</i> (2002), Waage <i>et al.</i> (2010)
<i>Thermococcus kodakaraensis</i> KOD1	pUD- series pTN(K)- series	<i>pyrF</i> marker recovery Simvastatin markers	<i>E. coli</i> <i>E. coli</i> and <i>Thermococcus</i> sp.	Suicide vector Suicide and expression vectors	Sato <i>et al.</i> (2003; 2005) Santangelo <i>et al.</i> (2008)
<i>Sulfolobus islandicus</i> , <i>S. solfataricus</i>	pAG21	Alcohol dehydrogenase conferring resistance to butanol and benzyl alcohol	<i>E. coli</i> and <i>Pyrococcus</i> origin from pGT5	Shuttle vector	Berkner and Lipps (2008)
	pEXS-series and pMJ-series	Hygromycin B marker and <i>pyrF</i>	<i>E. coli</i> and viral replicon from SSV1	Shuttle and integration vectors	(Berkner <i>et al.</i> , 2008; Angelov <i>et al.</i> , 2010)

Amp, β -lactamase (ampicillin resistance gene); Cat, chloramphenicol acetyltransferase; Ery, erythromycin resistance gene; Kan, kanamycin acetyltransferase; trpB, tryptophan synthetase; *pyrE*, orotate phosphoribosyltransferase; *pyrF*, orotidine 5'-monophosphate decarboxylase.

resistance marker. These should be coupled to more conventional characteristics such as segregational and structural stability, high transformation efficiencies (host and plasmid size dependent), a multiple cloning site and the blue/white screening strategy for rapid selection of successful ligations in *E. coli*. Most of the shuttle vectors summarized in Table 2 possess two antibiotic resistance markers (one for *E. coli* and the other for the thermophilic host). Together with the two origins of replication, this often results in large and therefore inefficient vectors. One of the few thermophilic shuttle vectors that reportedly utilized a single antibiotic resistance marker and single origin of replication for the Gram-negative *E. coli* and Gram-positive thermophilic host (*Geobacillus* sp.), pRP9 (De Rossi *et al.*, 1994), has been found to be difficult to transform and maintain in *E. coli* (Taylor *et al.*, 2008). This has been linked to the lack of a typical Gram-negative origin of replication (Mai and Wiegel, 2000) rather than the use of a resistance marker in both the mesophile and thermophile host.

It would also appear that although the presence of a thermophilic origin of replication is mandatory for autonomous replication in the thermophilic host, its source is less host specific. Origins of replication from thermophilic *Bacillus* spp. have functioned in *Thermoanaerobacterium* spp. (e.g. pRUKM) as have origins of replication from *Clostridium* spp. in *Thermoanaerobacter* spp. (e.g. pTE16). It is also evident that mesophilic origins of replication such as that in pUB110 work in *Geobacillus* spp. up to moderate temperatures, which is useful for the creation of T^s vectors. However, much remains unknown with respect to the applicability of common antibiotic resistance markers and origins of replication, e.g. the *Staphylococcus aureus* pCR194 chloramphenicol resistance marker, widely used in many of the plasmids described in Table 2, is apparently not expressed in *Thermus* spp. (Park *et al.*, 2004).

The major barrier to thermophilic vector development lies in the limited range of the suitable selective markers and agents (discussed separately). This limitation has often hindered developments in other areas, such as size reduction, structural integrity and general plasmid design. Vector design strategies have also focused particularly on the creation of 'suicide' vectors; plasmids that are unable to replicate autonomously in the thermophilic host (either through lack of a suitable origin of replication or temperature-dependent functionality of the plasmid) but contain homologous DNA to a particular gene or operon on the host organism. The homologous DNA typically flanks a thermostable marker and transformation of the host results in the isolation of single or double-cross-over mutants, where the selective marker is integrated at the target site in the genome (integration having occurred due to the presence of homologous sequence and selection

being based on the absence of a thermostable origin of replication in the plasmid). This has been used to disrupt genes and divert carbon flux but has also been used to insert promoters to 'upregulate' gene expression (Cripps *et al.*, 2009). For the engineering of *Geobacillus* spp., suicide vectors of the pTMO- series use a slightly different strategy, where the kanamycin marker is separate from the homologous DNA. Selection of kanamycin resistant single-cross-over mutants at the non-permissive temperature allows marker retrieval in the subsequent selection of double-cross-over mutants whereby the fragment of plasmid containing the marker 'loops out' from the genomic insertion locus, in some cases carrying a fragment of the original gene with it. This will leave the plasmid-borne, 'knockout cassette' in the genome resulting in a non-functional gene and gene product. Since selective markers and reagents are so significant to the successful application of molecular biology in thermophiles, their retrieval is invaluable for use in targeting other genes for knockout (the continuation of strain development).

Selective markers

Conventionally, identification of the presence of foreign DNA in a transformed host is verified by the inclusion of a gene, the product of which transfers a readily identifiable phenotype to the host strain. These selective markers are typically genes which impart antibiotic resistance (such as chloramphenicol acetyltransferase or the *cat* gene), but have also included genes which complement a particular nutritional deficiency in the host [such as plasmid-borne expression of the tryptophan synthetase gene (*trpB*) in auxotroph mutants of the genera *Thermus* and *Rhodothermus* (Koyama *et al.*, 1990; Bjornsdottir *et al.*, 2005; Bjornsdottir *et al.*, 2007)], impart resistance to toxic compounds or generate a visual signal (such as green fluorescent protein). High temperatures render many of these selective systems inappropriate, principally because of the thermal instability of the enzymes/proteins or the selective agents, i.e. ampicillin has a half-life ($t_{1/2}$) of only 3.3 h at 72°C.

The development of antibiotic-based selective markers has been the most actively pursued route in the development of temperature-stable vectors, despite many antibiotic compounds being intrinsically unstable at moderate to high temperatures. For routine work above 50°C, a few antibiotics are sufficiently stable to ensure reliable selection over 24–48 h [based on typically high growth rates of prokaryotic thermophiles under optimum conditions (Peteranderl *et al.*, 1990)]. They include kanamycin, neomycin (both demonstrating *no loss* of selectivity with incubation at 72°C and 50°C), chloramphenicol ($t_{1/2} = 40.6$ h at 72°C and 109.1 h at 50°C) and erythromycin ($t_{1/2} = 77.4$ h at 72°C).

The effects of temperature and pH on a range of antibiotics in different growth media have previously been reported in great detail (Peteranderl *et al.*, 1990). This study highlights that kanamycin was the only antibiotic of those described above, that demonstrated high stability > 50°C and no significant variability in selective potency when incubated at pH 5 or 7 and 50°C or 72°C (interestingly neomycin showed *increased* potency when incubated under certain conditions as compared with controls). These observations explain why kanamycin in particular is so widely chosen for thermophilic molecular biology work. Interestingly, the early reports of selection in thermophilic archaea relied on the previous generation of an auxotrophic mutant [often uracil-auxotrophic mutants with mutations in the orotidine-5'-monophosphate decarboxylase gene (*pyrF*)] (Sato *et al.*, 2003; Berkner and Lipps, 2008; Wang *et al.*, 2009). This may be due to the instability and hence redundancy, of kanamycin at temperatures as high as 85°C. As methods developed for this spp. antibiotics such as simvastatin (presumably highly stable at > 80°C) are now being used routinely, a major breakthrough that does away with the need for an auxotrophic mutant (Matsumi *et al.*, 2007; Waage *et al.*, 2010).

Other genetic methods of consideration

The vast majority of genetic methods thus far developed focus on those factors considered above, but other fundamental but as yet barely explored methods, require consideration. Transposon mutagenesis is a fundamental technique in discerning key genes in any biological system. Few examples exist of its application in thermophiles, the one notable exception being the use of the Tn916 transposon in both *Geobacillus stearothermophilus* and *Thermus aquaticus* (Sen and Oriol, 1990; Natarajan and Oriol, 1991), significantly in these examples, identified by means of co-integration of a functional amylase.

Temperature-dependent mobile group II introns may also have relevance to future, integrative technologies in thermophiles. These site-specific retro-elements were recently discovered in the cyanobacterium *Thermosynechococcus elongatus* and could be ideally suited to targeted gene knockouts in these thermophiles (Mohr *et al.*, 2010). Collectively, the methods for random insertional mutant generation may be of significant benefit in the development of desirable phenotypes in these and other industrially significant strains. To date and to the author's knowledge, a thermophilic knockout mutant library has not been reported, but may be a valuable resource of phenotypes in the case where site-directed genetic manipulation has failed or gives undesirable results.

Genetic methods underpin whole-cell biocatalyst development

Thermophilic ethanol producers

A number of recent papers have reported significant advances in the development of genetic systems in three thermophilic genera: *Geobacillus*, *Thermoanaerobacter* and *Thermoanaerobacterium* (Georgieva *et al.*, 2007; Shaw *et al.*, 2008; Cripps *et al.*, 2009; Taylor *et al.*, 2009). The genetic tools described in these reports have resolved many of the technical limitations which have prevented the development of these organisms as industrial 'cell factories'. The principal interest in these organisms is in the generation of ethanol, and particularly in the coupling of high-temperature fermentation and ethanol recovery by application of a gas stream and ethanol recovery facilitated at a higher temperature (Hartley and Sharma, 1987; Sommer *et al.*, 2004). While the concept evolved in the 1980s, the development of high-ethanol-yielding strains was hampered by the inability to eliminate organic acid production in the mixed acid fermentative host. However, over the past 5 years genetic tool development has led to successful pathway engineering strategies which have redirected carbon from organic acid to ethanol, principally through the targeted deletion and upregulation of genes. Specifically these include the deletion of lactate dehydrogenase and pyruvate formate lyase and the upregulation of the endogenous pyruvate dehydrogenase operon in *Geobacillus* spp., yielding commercially viable variants of *Geobacillus thermoglucosidarius* NCIMB 11955, the deletion of a [FeFe]-hydrogenase, acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 and lactate dehydrogenase in *Thermoanaerobacter mathranii* BG1 (Yao and Mikkelsen, 2010). In the former two examples carbon flux from glucose was directed at the pyruvate node, to ethanol through these selective mutations. In the latter example, glucose carbon flux was diverted in the same manner but the further expression of a *de novo* glycerol dehydrogenase increased ethanol production from glycerol as well.

The potential exploitation of high-temperature cellulose degradation

Biofuel production, specifically from biomass, currently attracts the most attention in this area of biotechnology. The ability to degrade cellulose and other complex polysaccharides is a feature of some prokaryotic thermophiles as well as a number of thermophilic archaea (Turner *et al.*, 2007). Genera known to possess these capabilities include members of the orders *Thermotogales* and *Clostridiales* (it is worth noting that a number of thermophilic species of the genus *Clostridium* have

been reclassified to *Thermoanaerobacter* spp. and members of other genera, with only *Clostridium thermocellum*, of the thermophilic cellulose degraders, retaining this genus name). Progress in the genetic modification of these genera need not be limited and given appropriate genetic development, the potential of these organisms for cellulose degradation and biofuels production (either ethanol or H₂), is considerable (Demain *et al.*, 2005). A number of plasmids and gene transfer systems specific for *C. thermocellum* have been reported and have been reviewed recently (Tyurin *et al.*, 2006). These may be transferrable to other thermophiles of the order *Clostridiales* which include *Caldicellulosiruptor bescii* (formerly *Anaerocellum thermophilum* DSM 6725), *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor obsidiansis* (van de Werken *et al.*, 2008), all of which may have potential for future thermophilic biofuel production beyond ethanol, i.e. H₂.

Members of the genus *Thermotoga* (principally *T. maritima*, *T. petrotoga* and *T. neapolitana*) have been reported to produce trace amounts of ethanol and higher yields of H₂ (Connors *et al.*, 2006). These species also typically possess the catabolic capacity to degrade xylan, cellobiose, xylose and pectin but not crystalline cellulose (Connors *et al.*, 2006). Although few reports of genetic manipulation of members of this genus exist, the potential for transformation of these species exists, based on plasmid transfer using the plasmid pRQ7 and its progeny shuttle vectors pJY1 and pJY2 (Table 1). Transient expression of the *cat* gene in both *T. maritima* and *T. neapolitana* was demonstrated in 2001 (Yu *et al.*, 2001), although the authors are not aware of further progress towards the development of a gene transfer system for these organisms. The need for development of such system is well understood and has been expressed repeatedly (Childers *et al.*, 1992; Connors *et al.*, 2006).

Industrial products

The development of thermophilic organisms for bioethanol production from cellulose has been emphasized. However, there is scope for their application in the production of other alcohols. A *Synechococcus* sp. has recently been engineered to produce isobutyraldehyde and isobutanol directly from CO₂, via the upregulation of ribulose 1,5-bisphosphate carboxylase/oxygenase commonly known as Rubisco (Atsumi *et al.*, 2009). Although it is a mesophilic organism, the genetic tools developed for its manipulation could be extended to the thermophilic variants such as *T. elongatus* and other thermophilic *Synechococcus* sp. for the production of these alcohols and other chemicals. A version of this technology is already sold by Sigma-Aldrich under the name TargeTron® for the manipulation of a variety of bacterial species including *E.*

coli, *Staphylococcus aureus*, *Clostridium perfringens*, *Shigella flexneri*, *Salmonella typhimurium* and *Lactococcus lactis*. These systems also have relevance to recent understanding of physiology in these strains, as discussed in the fundamental knowledge section of this review.

Compatible solutes are another group of biotechnologically important products for application in vaccine stabilization, as moisturizers in the cosmetics industry and inhalers for asthmatics. One of the best candidates for the production of these compounds has been the Gram-negative, obligatory aerobic, thermophile *Rhodothermus marinus* which accumulates mannosylglycerate under osmotic or temperature stress (Martins *et al.*, 1999; Roberts, 2005). This product has been commercialized under the name Firoin®, available from Sigma-Aldrich. In 2005 a genetic system for this bacterium was developed (Bjornsdottir *et al.*, 2005; 2006). Using this as a tool, the pathways for the production of mannosylglycerate as well as other industrially useful solutes accumulated by this organism (e.g. mannosylglyceramide and trehalose) could be engineered for improved production (Bjornsdottir *et al.*, 2005).

Contributions to fundamental knowledge

One of the ideal prokaryotic biological models for our understanding of thermophily is *Thermus thermophilus* reviewed in detail recently (Cava *et al.*, 2009). This species has contributed significantly to our understanding of genetic manipulation at high biological temperature as well as structural genomics and systems biology (Pantazaki *et al.*, 2002; Cava *et al.*, 2009). Commercially, this genus has been more a source of stable enzymes (Pantazaki *et al.*, 2002) rather than whole-cell biocatalysts, in spite of the fact that *T. thermophilus* has been characterized as mediating degradation of organic compounds such as xylan (Beffa *et al.*, 1996; Lyon *et al.*, 2000).

The relevance and work in this species which we wish to highlight has been where the establishment of a sound and fundamental knowledge base of physiology and genetic systems (Tables 1 and 2) has evolved to deliver not only many useful enzymes and bioproducts but also has assisted in developing biological models and understanding. *Thermus thermophilus* HB27 is of particular relevance to illustrate this point with observations of natural competency (Friedrich *et al.*, 2001) being linked to an unusual piliation phenotype (Friedrich *et al.*, 2002; 2003). Furthermore transformation by electroporation (de Grado *et al.*, 1999) and a variety of plasmids allowing chromosomal integration (Weber *et al.*, 1995; Tamakoshi *et al.*, 1999) and gene expression (de Grado *et al.*, 1999; Moreno *et al.*, 2003) have revealed fundamental methods of gene induction and DNA replication (Wayne and Xu, 1997; Ernstsson *et al.*, 2003).

Although being valuable tools for commercialization of thermophilic microorganisms, many of these genetic tools can also be applied to studies of high-temperature physiology and metabolic networks. A recent example of how the development of such tools has aided in the elucidation of such pathways is in the investigation of photosystem II in the thermophilic cyanobacterium *T. elongatus*. This membrane protein complex catalyses the first step in the light reaction of photosynthesis, which is the light-driven oxidation of water into molecular oxygen and protons (Sander *et al.*, 2010). The genetic tools for this organism have been in development for a number of years, with the first report of transformation of a thermophilic *Synechococcus* sp. in 1970 (Shestakov and Khyen, 1970). Initially, methods for transformation relied on the natural competence of *Synechococcus* sp., but later improvements included the use of electroporation techniques and the inactivation of a type I restriction endonuclease which prevented foreign DNA species from being established in these bacteria (Muhlenhoff and Chauvat, 1996; Iwai *et al.*, 2004). Methods such as these are also prompting strain development programmes, wherein pyruvate decarboxylase and alcohol dehydrogenase have been expressed in recombinant cyanobacteria, which are then capable of producing ethanol (US Patents 6 306 639 and 6 699 696). Similarly, the moderate thermophile *Chlorobium tepidum* has served as a model organism for the study of the evolution of photosynthesis. The development of a gene transfer system has allowed researchers to make a variety of knockout mutants which have contributed to the characterization of the carotenoid synthesis pathway (Wahlund and Madigan, 1995; Frigaard *et al.*, 2004a,b).

It is perhaps fitting to finish this section with a deeper consideration of the genetic systems of thermophilic archaea and in particular the genera *Thermococcus*, *Pyrococcus* and *Sulfolobus*. Significant advances have been made in the development of adaptable and effective genetic systems in all three of these genera but in particular *Thermococcus kodakaraensis* KOD1 and *Sulfolobus islandicus* and *Sulfolobus solfataricus*. An in-depth analysis of the genetic tools available for these spp. will not be made here since it would be fair to say that to date in the reported literature, they have not been used to develop strains with industrially relevant phenotypes. In addition the systems have been reviewed in detail elsewhere (Berkner and Lipps, 2008; Wang *et al.*, 2009). The significance of this work collectively is to highlight the importance of a diverse genetic toolkit in understanding fundamental physiology. This is perhaps best highlighted by the myriad of tools developed for *T. kodakaraensis* KOD1 which are now paying dividends in the form of the ability to generate deletion mutants, study their effects and better understand physiology as a result (Santangelo *et al.*, 2008; Wang *et al.*, 2009; Borges *et al.*, 2010; San-

tangelo and Reeve, 2010). This is not to say that these genera have no industrial application; far from it. The common incidence of hydrogenases and the ability to produce hydrogen (Kanai *et al.*, 2005; Jenney and Adams, 2008), coupled to relevant catabolic phenotypes (Leveque *et al.*, 2000; Niehaus *et al.*, 2000; Maurelli *et al.*, 2008), make them an attractive target to biofuel researchers, even more so with the established and developed genetic methods that already exist.

Suggestions for future approaches

Much of the work to date in this area has been to develop a set of tools in order to deliver a tangible end-product be it a whole-cell biocatalyst or bioproduct. Although this approach has certainly been successful and has contributed to the current pool of accessible genetic methods, protocols and plasmids, rigorous investigation into the more fundamental aspects of the molecular biology has not been of top priority. Questions with respect plasmid replication, selection and maintenance still exist and transformation protocols other than electroporation could be developed that combine an understanding of mechanisms such as natural competency and gene transfer with the need to develop a strain or express a gene. The approach undertaken in the understanding of similar systems in *Thermus* spp. has incorporated aspects of this approach and from this work; a broader picture of fundamental aspects of physiology has emerged. This review highlights a degree of synergy in the approaches taken for the development of separate genetic systems in different thermophilic prokaryotes but also speculates as to how biologically different are mesophilic and thermophilic mechanisms of transformation and plasmid maintenance. It is clear that the beginnings of a more general and broader understanding of high-temperature genetics is emerging, but more fundamental and empirical research is required to fully understand the mechanisms in place.

Conclusions

At long last the tangible value of thermophilic prokaryotes as whole-cell biocatalysts has been realized. This is due in no small part to the diligence and perseverance of researchers who have sought to develop stable, reliable and reproducible genetic systems for these strains. The rewards of perseverance have been plenty and include, among others, several commercial homoethanologenic organisms and increased yields in valuable bioproducts such as compatible solutes. Although the success stories are few, the experience they bring is perhaps of greater importance than the artefacts they have produced. The intimate understanding we now have with regards what systems work best for high-temperature genetics will lay the foundation for future developments of other strains,

developments that can be facilitated and accelerated because of our experiences and understanding. It is also clear that this expertise is having an impact in other scientific arenas such as the expansion of the fundamental knowledge of metabolism and physiology of thermophiles. The authors suggest that for any success to be achieved in the future development of thermophilic-based processes, a solid, reliable and well-thought-out genetic system must be of utmost consideration.

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