Extremophiles (2009) 13:213–231
DOI 10.1007/s00792-009-0226-6

REVIEW

Thermus thermophilus as biological model

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Received: 3 September 2008 / Accepted: 31 December 2008 / Published online: 21 January 2009

Abstract *Thermus* spp is one of the most wide spread genuses of thermophilic bacteria, with isolates found in natural as well as in man-made thermal environments. The high growth rates, cell yields of the cultures, and the constitutive expression of an impressively efficient natural competence apparatus, amongst other properties, make some strains of the genus excellent laboratory models to study the molecular basis of thermophilia. These properties, together with the fact that enzymes and protein complexes from extremophiles are easier to crystallize have led to the development of an ongoing structural biology program dedicated to *T. thermophilus* HB8, making this organism probably the best so far known from a protein structure point view. Furthermore, the availability of plasmids and up to four thermostable antibiotic selection markers allows its use in physiological studies as a model for ancient bacteria. Regarding biotechnological applications this genus continues to be a source of thermophilic enzymes of great biotechnological interest and, more recently, a tool for the over-expression of thermophilic enzymes or for the selection of thermostable mutants from mesophilic proteins by directed evolution. In this article, we review the properties of this organism as biological model and its biotechnological applications.

Keywords Thermophiles · *Thermus* · Laboratory model · Genetic tools

Communicated by T. Matsunaga.

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Introduction

Extremophiles and thermophiles

Prokaryotes adapt to an incredibly wide range of physico-chemical conditions, in many cases far away from those considered as "normal" from our anthropocentric point of view. Those organisms that grow under such exceptional or "extreme" conditions have been named "extremophiles". However, what should define the extremophilic character for a given microorganism should not be our perception of how unusual an environment is but the existence of a limited biological diversity where such organism grows. However, there is no agreement as to how such a limit in the biological diversity should be quantified.

Temperature has been one of the classic physical parameters used to separate normal or "mesophilic" organisms from those grouped as psychrophiles or as thermophiles. The psychrophiles group has been defined as including those organisms that grow optimally below 15°C. However, notice should be taken that environments with temperatures below 15°C include most of the sea water, and that biological diversity in such places (e.g. the Antarctic seas) is spectacular, and therefore a more convenient definition of what a psycrophilic organisms is should probably be reappraised. By contrast, environments that keep stable temperatures above 50°C, the limit usually described for an organism to be defined as thermophile, are not so abundant, as they are restricted to hydrothermal areas around the world. Sporadic heating of specific environments such as those produced by decomposition of organic matter or highly sun-heated water surfaces such as salines or shallow lakes has provided the appropriate environment for the evolution of many microorganisms that grow optimally between 50 and 60°C, but also able to

grow at temperatures below 50°C. Actually, this group of "moderate thermophiles" includes genera from several phylogenetic groups with representative species of mesophilic character (e.g. *Clostrium, Bacillus*).

In contrast to the relatively widespread character of "moderate thermophilia", the ability to grow optimally above 70°C is a much less common property, actually limited to the prokaryotic world. In most cases these organisms, known as "extreme thermophiles", are unable to grow below 50°C, a likely consequence of the stable character of the thermal environments where they evolved. Even more biologically restricted is the so-called "hyperthermophilic" character (optimum growth > 85°C). Only a few short-branched phylogenetic groups of the Bacteria and Archaea Domains can be considered as hyperthermophiles.

Thermophiles as source of enzymes

From the very first descriptions of thermophilic microorganisms, one of the major points of interest was the way their macromolecules were adapted to function at such high temperatures. Special attention was paid to their enzymes, most of which exhibited optimal activity around the optimal growth temperature of the organism. The fact that such "thermozymes" display associated increased resistance to denaturing chemical agents (Li et al. 2005; Vieille and Zeikus 2001), prompted the chemical industry to investigate their specific applications in biotransformation processes or even as components of their final products. Nevertheless, the major turning point in the potential applicability of thermozymes was the description of the use of the DNA polymerase of *Thermus aquaticus* in the polymerase chain reaction (PCR), and further derived techniques, a fundamental step in the explosive development of our biological knowledge.

The production of thermozymes is usually carried out on surrogate mesophilic hosts, from which the protein can be easily purified by its differential thermal stability with respect to most of the proteins from the host. However, this method is usually limited to simple monomeric or homo-oligomeric soluble enzymes that do not require specific cofactors, post-transcriptional modifications, proteolytic processing, suitable chaperones, or to form multiprotein complexes to be stabilized. Actually, in the course of the early structural genomic programs focussed on (hyper)thermophiles it was estimated that less than 20% of the ORFs in any genome would likely be expressed as a stable, properly folded protein, in *Escherichia coli* (Jenney and Adams 2008). Therefore, the use of a genetically related and laboratory-friendly thermophilic host is a likely alternative to mesophilic host as cell factories for the production of thermostable proteins.

Reasons to work with thermophiles

In addition to the applicability of their enzymes, thermophiles have biological properties that make them highly interesting subjects for basic science. In structural biology, the enzymes and macromolecular complexes from extreme thermophiles have been selected as target models because they are easier to crystallize than their mesophilic counterparts. This empirical observation is most likely due to a tradeoff between thermostability and structural rigidity during adaptation to thermophilic environments. Paradigmatic examples of large complexes from thermophiles crystallized are the high-resolution structures of the 70S ribosome (Yusupov et al. 2001), the bacterial RNA polymerase (Selmer et al. 2006; Severinov 2000), or the respiratory complex I (Sazanov and Hinchliffe 2006) from *Thermus* spp, that were resolved before those of *E. coli*. Actually, this apparently greater ability to crystallize has been the main reason for the first "structural genomic" programs to choose an extreme thermophile or a hyperthermophile as model subject (Jenney and Adams 2008).

An additional interest of extreme thermophiles and hyperthermophiles originates from their phylogenetic position, as most of them belong to the deepest branches of their respective bacterial or archaeal phylogenetic trees (based in the sequence of the 16S rDNA and other molecules). Such phylogeny supports that these organisms could be the closest living representatives of the last bacterial or archaeal common ancestors (LCA), and, therefore, their study will reveal biological traits of primitive life on Earth.

Finally it is also relevant to point out the small size of the genomes of these organisms, most of which are below 2.5 Mbp. Either if such small sizes are a reminiscence of their ancient origin (the simpler, the older), or the consequence of the difficulties to preserve a high-fidelity replication process at high temperatures, the final result is that very few functional paralogues are found in the genomes of extreme thermophiles, thus making them good candidates to investigate the specific function of their genes through the isolation of directed knock out mutants.

Thermophiles as laboratory models

Despite the interest of extreme thermophiles and hyperthermophiles commented above, only a few of them can be regarded as tractable or at least promising laboratory models. The reasons for such scarcity of amenable thermophilic models are various, but most of them related to the high temperatures at which these organisms grow. Problems such as dehydration of the medium, the requirement for special solidifying components, requirement for glass plates and other thermo-resistant equipment, or the very same unstability of several growth medium

components are constitutively associated to the growth temperature of the organisms. Other problems not so evidents are associated to the metabolism of the selected model, in many cases requiring anaerobic growth conditions or low yield chemolitotrophic ways of gaining energy (Vieille and Zeikus 2001). Moreover, in a few cases the high temperature has to be combined with other extremophilic characters like in the case of *Natranaerobius thermophilus* that grows optimally at high temperature (53°C), high pH (9.5), and high salt concentration (3.3–3.9 M Na^{+}) (Mesbah et al. 2007).

To date, only the extreme thermophilic Archaea and Bacteria shown in Table 1 have been subjected to noticeable processes of genetic manipulation. Among the Archaea two species of *Sulfolobus* (Contursi et al. 2003; Jonuscheit et al. 2003; Worthington et al. 2003; Albers et al. 2006), and another two of *Pyrococcus* (Aravalli and Garrett 1997; Watrin et al. 1999; Lucas et al. 2002; Watrin et al. 1999) can be manipulated to a certain degree, whereas *Thermococcus kodakarensis* (Sato et al. 2003) has emerged in the last years as one of the most effective laboratory models of its kind. This was due to the parallel development of a transformation system and appropriate selection methods that allow yields around 10^2–10^3 transformants/μg of DNA. The selection methods for *T. kodakarensis* are based on the complementation of auxotrophic mutants for uracil or tryptophan (Sato et al. 2005), and in the use of Simvastatin, a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, which is an essential enzyme for archaeal membrane lipid biosynthesis (Matsumi et al. 2007). Actually, a series of selection/counter-selection steps based on the uracyl synthesis pathway and the use of 5′-fluoroorotic acid as counter-metabolite can be carried out to obtain series of deletion mutants of genes in this organism (Sato et al. 2005), allowing an in deep analysis of its biology.

Among extreme thermophilic bacteria, some attempts were made to manipulate strains of *Thermotoga maritima* with no further continuity in the literature (Yu and Noll 1997; Yu et al. 2001). By contrast, a few strains of the species *Thermus thermophilus* appear as really suitable genetic models and even excellent candidates for their specific application as cell factories or as hosts for the selection of thermostable mutants by directed evolution. The existence of great differences regarding genetic manipulation even among strains of the same species is most likely related to differences in the efficiencies of their competence and restriction systems (de Grado et al. 1999). In this review we will describe our present knowledge on the genetics and biotechnological applications of this genus.

The genus *Thermus*

Hundreds of strains from the genus *Thermus* (Brock and Freeze 1969) have been isolated from neutral or slightly basic thermal effluents all around the world, from self

Table 1 Extreme thermophiles as laboratory models

Organism	Top	Tran	Plas.	Virus	Antib. Resist.	Aux. Compl.	Rep. Gen.
Sulfolobus solfataricus	80	EP	++	++	Hyg	*pyrEF*	*lacS*
		CJ			Adh		
Sulfolobus acidocaldarius	80	CJ	+	+	−	*pyrEF*	−
		$CaCl_2$					
Pyrococcus furiosus	96	$CaCl_2$	+	−	Adh	−	−
Pyrococcus abyssi	103	PEG	+	++	−	*pyrEF*	−
Thermococcus kodakarensis	85	$CaCl_2$	+	−	Sim	*pyrF*	*lacS*
		NC				*trp*	
Thermotoga neapolitana	80	PEG	±	−	−	−	−
Thermus thermophilus	75	NC	++	+++	Kan		
		EP			Bleo	*leuB*	*bgaA*
		CJ			Hyg	*trpB*	*phoA*
					Str	*pyrEF*	*sGFP*

Top optimum temperature, *Tran* transformation methods, *EP* electroporation, *CJ* conjugation, $CaCl_2$ calcium chloride and thermal shock, *PEG* spheroplast and polyethylen glycol mediated transformation, *NC* natural competence, *Plas* plasmids transference described, *Virus* described viruses for the strain, *Antib. Resis.* Genes for thermostable resistance to antibiotics (Hyg, Kan, Bleo, Str, and Sim confer resistance to Hygromicin B, kanamycin, Bleomicin, Streptomycin, and Simvastatin) or antimetabolites (Adh, codifies for a resistance to alcohols), *Aux compl* complementation of auxotrophic mutants (*pyrE* or *pyrF*, for uracil, *leuB* and *trpB* for leucine and tryptophan, respectively), *Rep. Gen.* reporter genes (*lacS* and *bgaA* encode beta-galactosidases and *phoA* a hyperalkaline phosphatase). Symbols: − undescribed, ± Transitory presence; + to +++ Frequency of descriptions

heating piles of organic matter or even from industrial heating systems. All of them are thermophilic Gram negative bacteria that grow aerobically with high growth rates and good cell yields on complex medium at optimum temperatures ranging from 62 to 75°C, but do not require specific amino acids or vitamins. Under anaerobic conditions, some *Thermus* spp strains can use nitrogen oxides or even metals to grow by anaerobic respiration (see later). Most strains have an orange- or yellow-coloured because of the presence of a relevant fraction of carotenoids in their membranes. Morphologically they are slender bacillar-shaped cells which tend to form septated filaments in exponential cultures on rich medium, and thereafter separate by binary fission when reaching stationary phase. When grown in rich medium under low stirring conditions some strains form fragile multicellular structures known as "rotund bodies". No motile forms have been described.

Different *Thermus* species can adapt and grow in media containing up to 6% NaCl through the accumulation of compatible solutes. In *T. thermophilus*, the different behaviour of some of the strains under salt stress conditions result from the ability to accumulate trehalose, mannosylglycerate (MG), or both (Nunes et al. 1995), which ultimately depends on the presence of genes for the respective biosynthetic pathways (Alarico et al. 2005). Recent results indicate that MG serves as a compatible solute primarily during osmoadaptation at low levels of NaCl, while trehalose is primarily involved in osmoadaptation at higher NaCl levels (Alarico et al. 2007).

Another remarkable feature of *T. thermophilus* biology is its rare cellular polyamine composition that, in addition to standard polyamines (putrescine, spermidine and spermine), includes long (caldopentamine and caldohexamine) and branched (tetrakis (3-aminopropyl) ammonium) polyamines, which are rarely found in moderate thermophiles or mesophiles. This distribution suggests important roles for these unique polyamines in biochemical reactions at high temperatures. In this regard, such polyamines have been reported to be essential for protein synthesis at high temperatures as they are required for the formation of proper structure of the initiation complex between 30S ribosomal subunit, the messenger RNA, and the initial aminoacyl-tRNA. It has been also shown that polyamines stabilize RNA and DNA (Oshima 2007; Terui et al. 2005).

Several comparative studies on 16S RNA and protein sequences indicate that *Thermus* spp is closely related to the genus *Deinococcus*, forming an independent phylogenetic branch of the bacterial tree (Weisburg et al. 1989; Griffiths and Gupta 2004, 2007; Omelchenko et al. 2005). However, the phylogenetic position of this *Deinococcus-Thermus* phylum is still a matter of discussion. It was originally proposed that this phylum constitutes one of the oldest groups of the Bacteria Domain after those of *Thermotoga* and *Aquifex* on the basis of comparisons of 16S rRNA sequences (Woese 1987) and of conserved proteins. However, an alternative interpretation based on the presence of specific insertions and deletions (Indels) in proteins and on the complexity of the cell envelope supported an intermediate position for this phylum between the Gram Positives (Monoderms) and Gram negatives (Diderms) (Gupta 2000). More recently, the availability of complete genomes of *Deinococcus* and *Thermus* spp has allowed a wider analysis of the phylogeny of the group as those based on the concatenation of conserved orthologs (Ciccarelli et al. 2006) or on Clusters of Orthologous Groups of proteins (COGs) that supports the latter hypothesis as the most likely origin of the *Deinococcus-Thermus* phylum (Omelchenko et al. 2005).

The genomes of two strains of *T. thermophilus* are already available (Henne et al. 2004; Masui et al. 2005). In both cases the genome consists of a chromosome and a megaplasmid of 1.9 and 0.23 Mbp, respectively. The G + C content is quite high (69%) and the coding density is also high (95%, 2,200 proteins) (see Henne et al. 2004; Lioliou et al. 2004; Liebl 2004).

Envelope ultrastructure

The cell envelope of *T. thermophilus* appears as a complex pattern of four layers under the electron microscope (Castón et al. 1993) (Fig. 1a). The lipids of several strains of *Thermus* spp have been shown to contain two major components: a strain-specific glycolipid (a tetrasaccharide structure linked to a glycerol or to a long-chain diol in *T. thermophilus* Samu-SA1) and an apparently conserved glycophospholipid (containing a *N*-acylglucosamine and a *N*-glyceroyl-heptadecaneamine) similar to that found in *Deinococcus* (Huang and Anderson 1989; Anderson and Huang 1992). The exact location of these compounds in the envelope is not completely understood, by they could be part of the outer membrane (OM) that these organisms have. The principal fatty acids of the membranes are C15 and C17 branched-chain compounds (Pask-Hughes and Shaw 1982; Lu et al. 2004).

Surrounding the cytoplasmic membrane, a thin layer of peptidoglycan exists. Its basic monomeric subunit consists of *N*-acetylglucosamine–*N*-acetylmuramic acid–L-Ala–D-Glu–L-Orn–D-Ala–D-Ala, acylated at the δ-NH_2 group of Orninite by a Gly-Gly dipeptide. The last of these Gly residues is replaced by phenylacetic acid in a significant proportion (23%) of the muropeptides in *T. thermophilus*, but not in other species of the genus (Quintela et al. 1995). Thus, the murein composition and peptide cross-bridges of *Thermus* are typical for a Gram positive bacterium, but the

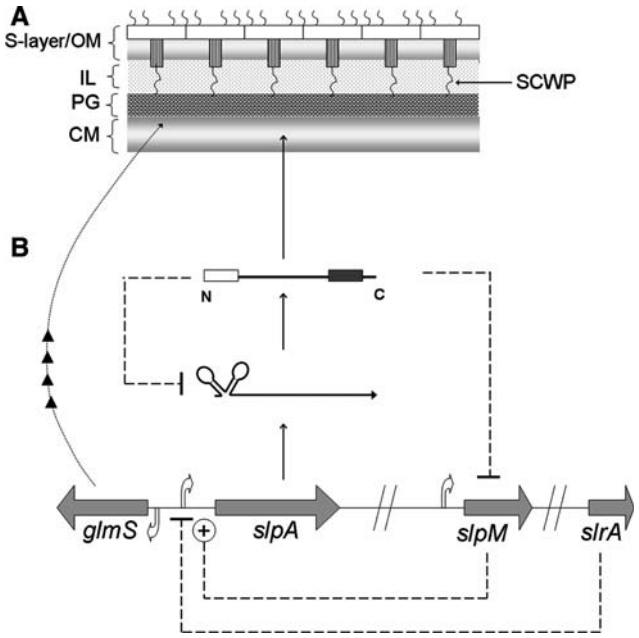

Fig. 1 Cell envelope structure and S-layer synthesis in *T. thermophilus*. **a** Scheme showing the different layers of the cell envelope of *T. thermophilus*. *CM* cytoplasmic membrane, *PG* Peptidoglycan, *IL* Intermediate amorphous layer containing the secondary cell wall polymers (SCWP) covalently associated to the peptidoglycan and bound through the SLH domain to the S-layer; S-Layer/OM) The S-layer-Outer membrane complex. **b** SlpA regulation. The genes coding for the S-layer (*slpA*), the glucosamine synthase (*glmS*) and the SlpM and SlrA proteins are shown as *grey arrows*. Transcription of *slpA* generates a mRNA with an untranslated leader region predicted to form an stable secondary structure. The mRNA is translated to an SlpA protein that contains a SLH motif (*white box*) and a domain implicated in mRNA binding (*grey box*). If the protein is not properly secreted, translation is arrested by binding of SlpA to the mRNA. Further data support that SlpA also represses transcription of *slpM* either directly or in an indirect way. In vitro evidences also support that SlrA and SlpM are able to bind the *slp*A promoter, with opposite effects on its transcription. GlmS catalyzes the synthesis of glucosamine. Its presence in a divergent operon with respect to *slpA* points to a putative coordination point in the synthesis of the cell wall and the S-layer of *T. thermophilus*

murein content, degree of cross-linkage, and glycan chain length are features more commonly found in those Gram-negative organisms so far studied, a fact that could explain the Gram-negative staining of the genus. It is also noticeable that the peptidoglycan of *Deinococcus radiodurans* shows a similar composition in its monomeric subunit (Quintela et al. 1999) despite its Gram positive staining.

The peptidoglycan layer is surrounded by an amorphous material that could correspond to the "intermediate" layer described for *D. radiodurans* (Baumeister et al. 1981). The composition of this material is not known yet however, it has been characterized by biochemistry and immunomicroscopy techniques as a secondary cell wall polymer (SCWP) covalently bound to the peptidoglycan (Cava et al. 2004a). Concomitantly, this SCWP is required for the attachment of a regular array of the SlpA protein (S-layer) through a N-terminal SLH (S-Layer Homology) domain suggesting an important role for this intermediate layer to preserve the structure of the cell envelope (see below). The presence of SLH domain mediated attachment of S-layers or secreted proteins to the cell wall is another property shared with Gram positive bacteria. Actually, and despite the enzymes responsible for the synthesis of this SCWP are not known, a homologue to a SCWP-pyruvylating activity from *Bacillus anthracis* (*csaB*) was identified in *T. thermophilus*. This enzyme performs the pyruvylation of the SCWP which ultimately leads to a high affinity attachment of the SlpA protein array to the cell wall. Therefore, as a consequence of a *csaB* mutation, the external envelope made of the OM and the S-layer (S-Layer/OM, complex) detaches and separates from the cell wall, leading to the formation of multicellular Bodies (MB) (Cava et al. 2004a), similar to the rotund bodies described for certain strains of *Thermus* spp (Brock and Freeze 1969). It is worth to mention that these MBs are apparently formed when the interactions between the cell wall layers become weaker or when their relative synthesis is unbalanced (Castán et al. 2001).

Noteworthy, the SlpA protein presents different traits from those S-layer proteins of other bacteria. Actually, its sequence shows characteristics more similar to the so-called "regular outer membrane proteins" (rOMP) than to other S-layer (Engelhardt and Peters 1998). These includes its insoluble character, the ability to form porin-like crystals in the presence of detergents when the peptidoglycan has been digested (Castón et al. 1993), and the low accessibility to antibodies in vivo (unpublished results). However, despite these differences with other S-layer proteins, SlpA forms hexagonally arranged crystals both in vivo and when purified in the presence of peptidoglycan (Castón et al. 1993). Interestingly, an additional protein (SlpM) overexpressed in *slpA* mutants forms S-layer like hexagonal lattices (Olabarría et al. 1996). The role of this protein in vivo is not known, but the fact that it is expressed in low amounts in the wild type strain and that *slpM* mutants still show an hexagonal S-layer makes unlikely any role for this protein in the formation of the S-layer in wild type cells as it has been suggested (Engelhardt and Peters 1998). Overall, these data support that SlpA forms an hexagonal array in vivo that acts as scaffold for the OM, attaching it to the cell wall through the binding of its SLH domain to the secondary cell wall polymer (SCWP). In this sense, the SlpA protein could play a role as spacer and linker similar to that of the Ompα from *T. maritima* (Engel et al. 1992). Besides SlpA, the protein composition of the OM is not known, but porins (Maier et al. 2001), components of the competence apparatus (Rumszauer et al. 2006), and an Omp85-like protein of the

protein integration apparatus (Nesper et al. 2008) have been described as OM components of *T. thermophilus.*

The expression of SlpA is tightly regulated (Fig. 1b). The *slpA* gene is transcribed within a monocistronic mRNA from a strong promoter negatively regulated by the SlrA and the SlpM proteins (Fernandez-Herrero et al. 1997). Concomitantly, the *slpM* gene is under the control of SlpA, in such a way that, as stated above, *slpA* mutants over-express SlpM (Olabarría et al. 1996). In addition to this transcriptional control, SlpA is able to control its own translation by binding to the leader region of its mRNA (Fernandez-Herrero et al. 1997). Actually, deletion of this leader mRNA makes the cell to continue the expression of the SlpA protein in early stationary phase, leading to the formation of MBs (Castán et al. 2001). Further in vitro North-Western blot experiments with SlpA truncated derivatives revealed that a region close to the C-terminus of the protein was required for the binding to the mRNA leader (Castán 2004) suggesting a control mechanism by which any blockage in the SlpA secretion would trigger the repression of its own translation. However, the way by which SlpA also controls the expression of SlpM and vice versa is not yet understood.

It is also worth to mention the presence of *glmS* in a *slpA*-divergent operon. GlmS catalyzes the synthesis of glucosamine, the first precursor in the synthesis of the glycan moiety of the peptidoglycan. Despite the absence of evidences it is tempting to speculate about a putative coordination of the transcripction of both genes as a point of control of the cell envelope synthesis.

Despite this complex regulation, the *PslpA* promoter is extremely useful for the expression of genes encoding thermostable resistance to antibiotics in both *Thermus* spp and in *E. coli* (see below).

Metabolism

Thermus thermophilus uses various proteinaceous substrates as well as carbohydrates for growth. These are made available by numerous (exo) proteases, lipases, pullulanases, glucosidases and galactosidases encoded in its genome (Henne et al. 2004). For substrate uptake, the organism preferably uses energy-coupled systems, as at least 42 complexes are primary transporters belonging to the ATP-binding cassette (ABC) protein family and no homologues to phosphotransferase systems are apparently encoded in its sequence. Biosynthetic pathways for all 20 amino acids are also encoded in *T. thermophilus* HB27 as well as the genes for gluconeogenesis and for the synthesis of vitamins and cofactors such as folate, biotin, riboflavin, molybdopterin, thiamine, panthotenate, porphyrins and carotenoids. In some cases (e.g. the biosynthesis of cobalamin and carotenoids) the terminal steps of these biosynthesis pathways are encoded on a large plasmid (pTT27), whereas precursor synthesis is accomplished by enzymes encoded in the chromosome (Henne et al. 2004).

Regarding catabolic pathways, *T. thermophilus* encodes the information to metabolize most amino acids, as well as the genes for a complete urea cycle, the β-oxidation pathway in multiple copies, a complete tricarboxylic acid cycle and the glyoxylate bypass are also encoded in the genome (Henne et al. 2004). ATP synthesis is accomplished by an ATP synthase of the V/A type, which refers to the special phylogenetic position of the *T. thermophilus* complex between A0A1-type and V0V1-type ATPases (Esteban et al. 2008; Toei et al. 2007; Yokoyama et al. 1994, 2000a,b). Actually, it has been proposed an archaeal origin for this V/A type of ATPase in *T. thermophilus* by horizontal gene transference (HGT) (Müller and Gruber 2003). However, typical bacterial F-ATPases have been detected by biochemical means (subunit composition and specific antibodies reactivity) in two isolates of the genus, *T. scotoductus* SE-1 and *T. filiformis* (Radax et al. 1998), supporting a bacterial origin for the corresponding genes in these strains through independent HGT events.

Aerobic respiration

Despite the low solubility of oxygen at high temperatures, most strains of the genus have been isolated by aerobic growth, and actually, oxygen appears as the only electron acceptor that supports growth in many strains (da Costa et al. 2001). In fact, the respiratory enzymes of *Thermus* have been the subject of several biochemical and structural studies, making its aerobic electron transport chain one of the best characterized (Fig. 2a) (Mooser et al. 2006). Most reducing equivalents from the catabolic metabolism are fed into the electron transport chain (eTC) via the proton-translocating Type I NADH dehydrogenase (NDH-1) (TTC1907–1920, *nqoA-N*) that resembles the mitochondrial complex I (Yano et al. 1997). The 3D structure of the soluble domain of this enzyme has been published (Sazanov and Hinchliffe 2006). In addition, electrons could enter the eTC through the succinate dehydrogenase or complex II (TTC1089–1092), and putatively also through a monomeric quinone-dependent type-II NDH (TTC1829) (Yagi et al. 1988). A nicotinamide nucleotide transhydrogenase (TTC1778–1780) might act as a respiratory chain-linked proton pump (Park et al. 1992; Stupak et al. 2006). Two terminal cytochrome oxidases have been described for *Thermus*: a caa_3- type (TTC1670–1673) expressed under high oxygen pressure, and a ba_3-type oxidase (TTC768–770) expressed under limited O_2 supply (Fee et al. 1993; Hellwig et al. 2002; Mooser et al. 2006). The 3D structure of the *ba3* oxidase is already available (Soulimane et al. 2000), and evidences exist to support that a periplasmic

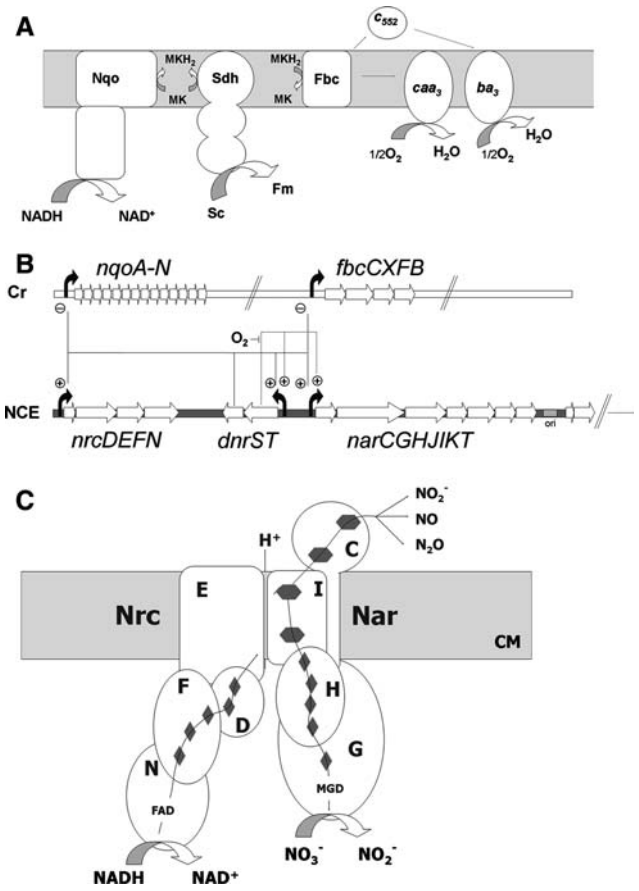

Fig. 2 Aerobic respiration and denitrification in *T. thermophilus*. **a** Scheme of the aerobic eTC of *T. thermophilus* in which the respiratory complexes I (Nqo), II (Sdh) and III (Fbc) are indicated along the periplasmic cytochrome c_{552} and the final caa_3 and ba_3 oxidases. Menaquinone-8 (MK) is the main component of the quinone pool. *Small arrows* indicate the electrons pathways. **b** Scheme of the regulation performed by DnrS (*dotted lines*) and DnrT (*continuous lines*) for the expression of the NCE operons (*grey bar*) and for two of the main operons of the aerobic respiratory eTC encoded in separated loci of the chromosome (Cr). **c** NarC and NarI constitute a respiratory supercomplex where NarC rules the deviation of the electrons towards the Nir, Nor and Nos reductases depending on the availability of nitrogen oxides. In the absence of nitrate, electrons will flow from the external heme *b* of NarI to the heme *c* groups of NarC to finally reach the denitrification reductases. + transcriptional activation, − transcriptional repression, ori NCE replication origin, O_2 Inactivation of DnrS by oxygen, *CM* cytoplasmic membrane, diamonds Fe–S centers; hexagons *heme c* and *b* groups

cytochrome c_{552} (*cyaA*, TTC1058) serves as its electron donor (Muresanu et al. 2006). In contrast, the caa_3-oxidase could receive electrons directly from the recently described complex III (TTC1567–1570, FbcCXFB) (Janzon et al. 2007; Mooser et al. 2005).

Denitrification

Denitrification is an anaerobic respiration process present in several anaerobic and facultative bacteria by which nitrate is reduced to N_2 in four consecutive steps (Zumft 1997). There are facultative strains of *T. thermophilus* that can grow anaerobically by partial or complete denitrification (Cava et al. 2008c). In the first case, nitrate is reduced to nitrite that accumulates in the medium as the final product. This process was shown to be codified by a DNA fragment that could be transferred by conjugation to an aerobic strain, allowing the receptor to grow anaerobically (Ramírez-Arcos et al. 1998). This DNA fragment, named nitrate respiratory conjugative element (NCE), codes not just for the expected terminal nitrate reductase (Nar), but also for a new type of respiratory NDH (Nrc), and for the regulatory elements involved in their expression (Cava and Berenguer 2006). A cryptic replicative origin is also located immediately downstream of the *nar* operon, and the still unidentified origin for conjugative transference seems to be downstream and apparently not far from the *nar* operon (Ramírez-Arcos et al. 1998). Therefore the NCE constitutes a self-mobilizable 'nitrate respiratory island' widely conserved in denitrificant strains of *T. thermophilus* isolated from different parts of the world (Cava et al. 2008c). The phylogenetic analysis of the Nar and Nrc sequences supports an ancient origin for both enzymes, in agreement with the phylogenetic position of *Thermus* spp. deduced from its 16 S RNA sequence (Cava et al. 2004b; Philippot 2002).

In addition to the basic components of the nitrate reductase present in most bacteria (*narGHJI)*, the *nar* operon from *T. thermophilus* (*narCGHJIKT*) codes for two homologues to nitrate/nitrite polytopic membrane transporters (NarK and NarT) of the major facilitator superfamily (Ramïrez-Arcos et al. 2000) and for a dihaem cytochrome *c* (NarC), which first half shows high sequence similarity to the periplasmic cytochrome c_{552}. NarC constitutes the fourth subunit of this unique heterotetrameric enzyme, being its presence essential for membrane attachment and maturation of the enzyme (Zafra et al. 2002, 2005).

The four-gene operon *nrcDEFN* (for nitrate respiration chain) is located upstream of the *nar* operon (Fig. 2b). Three of these genes encode homologues to electron transporters of respiratory chains: NrcD to ferredoxins, NrcF to the iron–sulphur subunit of succinate:quinone oxidoreductases (SQRs) and NrcN to type-II NDHs. The Nrc proteins also form a heterotetrameric complex anchored to the cytoplasmic membrane through the NrcE subunit, a polytopic integral protein with a large (120 amino acids) C-terminal cytoplasmic domain where other subunits attach (Cava et al. 2004b). Functionally, this Nrc complex constitutes a new type (type-IV) of respiratory NDH, more similar from a structural point of view to the SQR than to the other NDHs. It is not known whether or not this nitrate-specific NDH can extrude protons during NADH oxidation.

Expression of the *nar* and *nrc* operons requires oxygen depletion and the presence of nitrate (Cava and Berenguer 2006). The NCE encodes two homologues to bacterial transcription factors in the bicistronic operon *dnrST* (Cava et al. 2007). DnrS is the oxygen sensor of the system, being required for the activation of the *nar* and the *dnr* operon promoters under anaerobic conditions. In contrast, DnrT is an oxygen-insensitive transcription factor of the CRP family that is required for the transcription of the *nar*, *nrc* and *dnr* operons (Cava et al. 2007). DnrT is able to function as activator of the *nrc* promoter when expressed aerobically from a constitutive promoter and also in aerobic in vitro assays (Cava et al. 2007). Thus the DnrT activity as transcription activator basically depends on its expression levels. Noteworthy, DnrT also represses the transcription of *nqo* (Cava et al. 2007) and *fbc* (Cava et al. 2008b), the operons coding for the respiratory complexes I and III of *T. thermophilus*, respectively. Despite the structure of DnrT has not been determined yet, we can not discard the putative existence of a metabolite bound to its N-terminal domain.

Thus, the NCE takes the control of the respiratory metabolism of *T. thermophilus* by replacing the aerobic eTC with a simpler one made of Nrc and Nar during nitrate respiration. It is noteworthy that such an effect requires the absence or reduced oxygen concentrations and the presence of nitrate, indicating that a two-signal integration system coordinates their expression, in a similar way to that described for other denitrifying bacteria (Zumft 1997).

Whereas only nitrate triggers the expression of Nrc and Nar in the partial denitrificant NAR1 strain, their expresion is induced by nitrate, nitrite and NO in complete denitrifying strains of *T. thermophilus*. Actually, in these latters, Nrc becomes the major electron donor towards the terminal reductases of the denitrification (Cava et al. 2008c). Interestingly, the menaquinone-oxidating role usually played by complex III in other denitrificant bacteria is assumed by the unusual cytochrome *c*-containing nitrate reductase in *T. thermophilus* (Cava et al. 2008b). Evidences exist to support that Nrc and Nar form a respiratory supercomplex during denitrification that uses NarC as a branching point to deviate electrons towards the Nir, Nor and Nos reductases of the denitrification apparatus depending on the nitrogen oxide available (Cava et al. 2008b). Therefore, being consistent with their energy hierarchy, if enough nitrate is present the electrons flow will be directed from the external heme *b* through the inner heme *b* of NarI and next to NarH and NarG to finally reduce nitrate inside the cytoplasm. However, if there is not enough nitrate, the internal *heme b* will be reduced and the electrons will bypass the nitrate reductase sink and flow from the external heme *b* of NarI outwards through one or both *heme c* groups of NarC to finally reach the Nir, the Nor or the Nos reductases (Cava et al. 2008b). In this sense, the heme *b* group of NarI could constitute the branching point for the Nar enzyme to function as a nitrate reductase or as a *bc*-like electron transport complex.

A model for structural biology

In the late 1990s, an international effort known as structural genomics (SG) was initiated to determine the 3D structures of representatives of all known protein families (Jenney and Adams 2008). The original SG began at RIKEN in Japan (Yokoyama et al. 2000a,b) where the initial focus was exclusively on the extremophiles and in particular *T. thermophilus* (Tt), which was among the first organisms to be targeted. Proteins from thermophiles were prime targets in the initial stages of the SG projects mainly because enzymes and macromolecular complexes of thermophiles are more easily purified, manipulated, and more stable during the long time periods needed for crystallization than those from mesophiles. Actualy, *T. thermophilus* has rendered 3D structures (Sazanov and Hinchliffe 2006; Selmer et al. 2006; Severinov 2000; Yusupov et al. 2001) which provides clues to discern the biological significance of many general processes shared by all living organisms. As an example of this, our present knowledge of the activity and motions of the ribosome during translation is mainly due to the high-resolution structures of the large and small subunits and of the complete 70S ribosome (Yusupova et al. 2001; Pai et al. 2008; Laurberg et al. 2008; Kaminishi et al. 2007; Connell et al. 2007; Selmer et al. 2006).

Moreover, the existence of genetic tools to manipulate *T. thermophilus* has led to the groups at Osaka University and RIKEN go further the SG program by developing the "Structural and Functional Whole-Cell Project for *T. thermophilus* HB8" (the reader is referred to the URL http://www.thermus.org for a more detailed description of this program). This "Structural and Functional Whole-Cell Project" pretends to understand the mechanisms of all the biological phenomena occurring in *T. thermophilus* by investigating the cellular components at the atomic level on the basis of their three-dimensional structures (Yokoyama et al. 2000a,b). So far 1450 Tt ORFs have been heterologously expressed from the 2238 ORFs predicted in the *T. thermophilus* HB8 genome; 944 recombinant proteins have been purified and 682 have been crystallized. These have yielded 360 structures to date by these groups deposited in the PDB (http://www.pdb.org) although very few have been formally described in publications, and hence few have any degree of biochemical characterization. Such a huge number of structures obtained by this program, along with other 106 structures obtained by other

groups make *T. thermophilus* one of the best known organisms from the structural point of view (21% of its encoded proteins have rendered 3D structures). This and the actual possibility of testing structure-based hypothesis by using the genetic tools available, allows to predict a wealth of biologically relevant data from the use of *T. thermophilus* as model organism.

Genetic manipulation of *T. thermophilus*

Gene transference

Among the properties that permitted the development of genetic tools for *Themus* spp, the most important of all is the constitutive expression of a natural competence system in several strains. The physiology of the process has been analyzed in six strains of *Thermus* spp revealing that the natural transformation process is dependent on divalent cations and pH (Hidaka et al. 1994; Koyama et al. 1986). Further genetic analysis based on the availability of the sequence of *T. thermophilus* HB27 revealed that at least 16 genes were implicated in natural competence (Friedrich et al. 2001, 2002). Three of them (*comEA, comEC, dprA*) encode proteins similar to components of DNA translocators, four pilin-like proteins (PilA1, PilA2, PilA3, PilA4), a leader peptidase (PilD), a traffic-NTPase protein (PilF), an inner membrane protein (PilC), a PilM-homologue, and a secretin-like protein (PilQ). In addition to these homologues of competence proteins, four additional proteins (ComZ, PilN, PilO, and PilW) were detected with no homologues in the protein data banks. Based on these data and on those of immunolocalization, a model for the natural competence system of *T. thermophilus* has been proposed (Averhoff 2004). It is interesting to note that the rates of DNA incorporation through this system (40 kb/s and cell) are among the highest ever measured, pointing to the huge efficiency of the system (Schwarzenlander and Averhoff 2006). Moreover, the competence system is energy dependent and highly efficient with DNA from Bacteria, Archaea and Eukarya, pointing to a relevant role for this system in interdomain DNA exchange (Schwarzenlander and Averhoff 2006). Actually, the natural competence system of *T. thermophilus* has been suggested to be responsible for the presence in this genus of genes coding homologues to proteins from Archaea (Omelchenko et al. 2005).

Conjugation has been also demonstrated between strains of *T. thermophilus*. Actually, it was shown that a conjugative element, probably a chromosomically-integrated plasmid, encoding a nitrate reductase and a NADH dehydrogenase can be transferred from a nitrate respiring strain to an aerobic one, allowing the receptor to gain the ability to grow anaerobically with nitrate as electron acceptor. This DNA transference was resistant to DNase, and could also bring to the receptor cell any other gene marker in a time dependent manner in mating-interruption experiments (Ramírez-Arcos et al. 1998). Therefore, the conjugation system is similar to the DNA transference from Hfr strains of *E. coli*. However, there are no current molecular data on the putative proteins implicated in this conjugative transference.

At the time of writing this, there are not descriptions of DNA transference mediated by phages between *Thermus* species and no cryptic phages have been identified in the genome of the *Thermus* spp so far sequenced. Actually, there is a quite limited number of reports on phages infecting *Thermus* spp. The first reported phage was the tailed icosahedral dsDNA phi-YS40, which infected *T. thermophilus* HB8 (Sakaki and Oshima 1975). Other isolates were described more recently as the filamentous phage PH75 also infecting *T. thermophilus* (Pederson et al. 2001) as well as phage TS2126 infecting *T. scotoductus* (Blondal et al. 2005). However, the most extensive description of *Thermus* specific phages was published in 2006 in a work in which 115 new isolates, putatively belonging to the *Myoviridae, Siphoviridae*, *Tectiviridae*, and *Inoviridae* families, were obtained from alkaline hot springs in New Zealand, Russia and the USA (Yu et al. 2006). Despite some of these phages have been subjected to further characterization (Minakhin et al. 2008; Jaatinen et al. 2008), the phi-YS40 phage still remains a the most characterized among the *Thermus* specific phages at the molecular level (Naryshkina et al. 2006; Sevostyanova et al. 2007).

Plasmids and replication

Thermus spp often harbour one or more plasmids, which seem to confer no significant advantages to their hosts (Munster et al. 1985). Only a few have been used further to develop bifunctional *E. coli-T. thermophilus* vectors and replication studies are even less frequent. The first group of shuttle *E. coli/Thermus* vectors was derived from pTT8, a cryptic plasmid present in *T. thermophilus* HB8, which has become one of the best studied (Koyama et al. 1990a). This 9.3-kb plasmid replicates by a tetha mechanism and encodes eight proteins, three of which showing significant similarities to those of some plasmids from mesophiles (Aoki and Itoh 2007; Takayama et al. 2004). One of these genes encodes a protein similar to the replication initiator protein, Rep, of the ColE2-related plasmids. In these, the plasmid-specified Rep protein specifically binds to the 31 bp minimal replication origin and synthesizes a short RNA molecule (5′-ppApGpA-3′) that is used as a primer for initiation of DNA synthesis by host DNA polymerase. In pTT8, the sequence 5′-TGTGCCCC(A/G)(T/A)GCTG GTG-3′ was identified as the binding site of recombinant

pTT8 Rep protein, and most likely could be the actual origin of replication of this plasmid in contrast to that preliminarily predicted by comparison with ColE2-related plasmids (Takayama et al. 2004).

One of the most widely used plasmids, pMK18, was obtained by the isolation of the minimal replication region of a 16 kbp cryptic plasmid from *Thermus* sp ATCC 27737 (de Grado et al. 1998). This minimal replicon consists of a 1,798 bp region that encodes a 402 amino acids long protein (RepA) required for its replication. RepA shows partial similarity to the RepT protein encoded by the plasmid pTsp45s from *Thermus* sp. YS45 (Wayne and Xu 1997) and to the ORF35 and ORF7 of plasmids pL4C and pS4C of *Thermus* sp. 4C, respectively (Ruan and Xu 2007). DNA binding assays by recombinant RepA revealed a high affinity for an A + T rich DNA region internal to its own coding sequence (de Grado et al. 1998). This fact, together with the absence of relevant repeated sequences that could mimic the replicative origins of other plasmids, supported this gene-internal region as its replicative origin. Further analysis by electron microscopy revealed the presence of typical theta forms, supporting this type of replicative mechanism for the plasmid (unpublished results).

Rolling circle replicating plasmids use plasmid-encoded replication initiation proteins (Rep), which bind to the double strand origin (*dso)*, nick the positive (leading) strand of the plasmid DNA before the leading strand is synthesized. The lagging strand is further synthesized from the single stranded origin (*sso*) after a single stranded intermediate was released from the double-stranded plasmid (Khan 2005). Recently, the 1,965-bp plasmid pTA103 from *T. aquaticus* NTU103 has been described to replicate by a rolling circle mechanism (Chu et al. 2006). The plasmid encodes two open reading frames, one of which showing sequence similarity to a Rep protein from a group III rolling circle plasmid of *Sinorhizobium meliloti*. Putative *dso* and *sso* origins and a putative nick site were identified in silico, and ssDNA intermediates were detected experimentally.

In conclusion, most of the plasmids presently used as cloning vectors for *Thermus* spp replicate through a theta mechanism, as most plasmids from Proteobacteria do, but rolling circle replication, typical from plasmids of Gram positives, is also found in the genus.

Positive selection

In addition to DNA transference the development of genetic tools and protocols for the use of *Thermus* spp as model required the development of selection procedures of the transformed cells. Initially, the description of the transformation capability of *Thermus* spp was based on the use of total DNA from spontaneous Streptomycin resistant mutants (Koyama et al. 1986), but selection of the first plasmids was based on the use of genes that complemented auxotrophic mutants defective in the synthesis of Leu or Trp (Koyama et al. 1990a, b). However, these selection procedures were hampered because of the high recombination between homologous sequences presented by these organisms (Koyama et al. 1986).

A main breakthrough in the development of genetic tools for *Thermus* spp was the independent identification of thermostable mutants of the kanamycin nucleotidyl transferase from *Staphylococcus aureus* by two groups in 1985 and 1986 using for the first time a thermophile-based selection method (Liao et al. 1986; Matsumura and Aiba 1985). In both cases, the authors identified two thermostabilizing mutations (Asp80Tyr and Thr130Lys) which conferred enough thermostability to the protein to allow the growth of *Bacillus stearothermophilus* on kanamycin at 61 and 63°C, temperatures at which the wild type protein did not confer any substantial protection. Combination of both mutations in the same protein allowed selection of this Gram positive thermophile up to 70°C on kanamycin (Liao et al. 1986). A few years later, our group and that of J. Fee expressed this gene in *T. thermophilus* from native promoters, allowing the construction of the first generation of plasmids conferring a thermostable resistance to kanamycin for this organism (Lasa et al. 1992b; Mather and Fee 1992) and also implementation of gene-directed knock out technologies in *T. thermophilus* (Lasa et al. 1992a). Further developments of protein expression and promoter probe vectors based on the use of this resistance followed (de Grado et al. 1998, 1999; Maseda and Hoshino 1995; Wayne and Xu 1997). The thermostable kanamycin nucleotidyl transferase remained as the only antibiotic resistance gene marker for *Thermus* until 2005, when two articles described the thermoadaptation of resistances to Bleomycin and Hygromycin by directed evolution of the corresponding parental genes in *T. thermophilus* (Brouns et al. 2005; Nakamura et al. 2005). The use of these new makers is compatible with the kanamycin resistance, allowing for example the analysis of the effects of a given gene expressed from a Bleomycin-selected plasmid on the expression of a reporter expressed from a second Hygromycin resistant plasmid in a kanamycin-resistant genetic (mutant) background (Cava et al. 2007).

Negative selection

Despite such recent wealth of selectable markers, the isolation of specific insertion mutants in *T. thermophilus* based on their use could generate polar effects on the downstream genes of an operon. In most cases, this effect can be relieved by the use of selection cassettes lacking a transcription terminator inserted in the same transcription sense as the targeted gene (Zafra et al. 2002). This way, the

downstream genes of the operon are constitutively expressed, so it can not be used for example to deduce any putative role of the target in transcription. To avoid this, different methods have been tried to eliminate the selection marker from the mutants. The classical negative selection by penicillin relays on the fact that only those bacteria growing actively are killed by beta-lactam antibiotics, whereas non growing cells survive. This method was followed to obtain kanamycin-sensitive derivatives lacking the 5'UTR of the *slpA* gene from a kanamycin-resistant parental strain of *T. thermophilus* (Castán et al. 2001). However, the use of this method is quite complicated because it required several intermediate constructions with the corresponding selection or counter-selection procedures. A less complicated procedure based on the use of *pyrE* gene as selectable marker on a Δ*pyrE* genetic background was described by (Tamakoshi et al. 1997, 1999). The *pyrE* gene encodes the orotate phosphoribosyltransferase of the pyrimidine biosynthesis pathway, and uracyl auxotrophic (URA) *pyrE* null mutants can be positively selected on plates containing 5′Fluoroorotic acid (FOA). Thus, a target*::pyrE* insertion derivative of a Δ*pyrE* mutant can be selected in mineral medium without uracyl, and a further selection of a Δ*target*, Δ*pyrE* double mutant can be carried out in a medium containing uracyl and FOA. A similar method has been applied in the development of the *Thermococcus kodakaraensis* genetics (Sato et al. 2005).

More recently, a much simpler method based on the use of a dominant *rpsL* allele (*rpsL1*) conferring dependence from Streptomycin (Str) has been developed (Blas-Galindo et al. 2007). It encodes a ribosomal S12 double mutant (K47R/K57E) protein expressed from its natural promoter that confers Str-dependence even when expressed as a single copy from the chromosome in the presence of a wild type *rpsL* allele. This property allows the selection of replicative and suicide vectors on plates with Str and their counter-selection on plates without the antibiotic. In this regard, a target gene can be deleted after a single transformation with suicide vector derivatives carrying downstream and upstream flanking regions followed by consecutive selections first on plates with and then without the antibiotic, as it is shown for the isolation of Δ*narC* mutants (Blas-Galindo et al. 2007).

Gene reporters

In addition to the use of the kanamycin resistance as reporter for the identification of promoters (Maseda and Hoshino 1995) a few genes coding for easily assayable thermostable enzymes have been used as reporters in *T. thermophilus*. The first of them was a beta galactosidase from *Thermus* spp (Koyama et al. 1990b), which has been used further to construct promoter probe plasmids (Cava et al. 2007; Moreno et al. 2003). A further reporter gene encoding a beta-glycosidase from *T. thermophilus* HB27 (TTC0042) was used to detect the expression of DNA repair genes (Ohta et al. 2006). Other enzymes used or of potential use as reporters are the malate dehydrogenase from *T. flavus* that was used to assay the expression of gene promoters in *T. thermophilus* (Kayser et al. 2001), and two alfa-galactosidases, one from *Thermus* spp (Koyama et al. 1990b) and another from *Geobacillus stearothermophilus* further thermo-adapted to function in *T. thermophilus* (Fridjonsson et al. 2002). Finally, a periplasmic hyperalkalyne phosphatase has been also used to develop promoter probe plasmids (Moreno et al. 2003).

Localization tools

The fluorescent proteins are extremely useful tools to study the biology of mesophilic bacteria and eukaryotes (Prescott et al. 2006). Recently, a multiple mutant of the Green Fluorescent Protein from *Aequorea victoria* was isolated in *E. coli* by its ability to help the folding of proteins fused to it (Pedelacq et al. 2006). This mutant, named superfolder GFP (sGFP) is able to fold and hence to fluoresce properly upon expression in *T. thermophilus* at 70°C, thus allowing its use as a tool to trace the location of proteins at high temperatures in this thermophilic host (Cava et al. 2008a). As with mesophiles, it was possible to construct bifunctional *E. coli-T. thermophilus* vectors from which fusions between a target protein and the sGFP could be expressed. Moreover, the sGFP could be localized inside the periplasm of *T. thermophilus* when fused to the PhoA hyperalkaline phosphatase, a twin Arginine transporter (TAT)-secreted protein. Therefore, the sGFP seems an excellent tool to identify proteins secreted by this mechanism in *T. thermophilus*. Future developments on thermostable colour variants of sGFP are expectable (Cava et al. 2008a).

Biotechnological applications of *Thermus* spp

Enzymes and proteins of biotechnological interest

Enzymes and proteins from the genus *Thermus*, and thermozymes in general are good candidates for biocatalytical processes as they often present higher operational stability, and enhanced co-solvent compatibility. Additionally, when produced in mesophilic hosts, purification may be carried out to a great extent by a single step of heat denaturation. However, not all thermozymes are susceptible of being recombinantly produced and diverse molecular biology tools have been made available for the use of *T. thermophilus* as cell factory (see below).

As shown in Fig. 3 the number of patents concerning enzymes from the genus *Thermus* has increased over the past 5 years. Evidently, the most important enzyme "mined" from thermophilic microorganisms is DNA polymerase, covering approximately one-third of all *Thermus* related patents filed between 2004 and 2008 (Fig. 3b) and with an estimated market of more than $350 million (€282 million) in 2005 and growing (http://www.in-pharmatechnologist.com/Materials-Formulation/Sigma-Aldrich-launches-DNA-Polymerase-kit). Ever since the original *T. aquaticus* polymerase was launched into the market, there has been interest for more processive, more reliable, stable and specific DNA polymerases. To that extent, two approaches have been developed: mining from other *Thermus* species or protein engineering on well-known polymerases. Regarding the former category, DNA polymerases have been reported and patented from *Thermus* sp., *T. filiformis, T. scotoductus, T. antranikianii, T. eggertssonni, T. kawarayensis, T. oshimai,* and *T. igniterrae* since 2003.

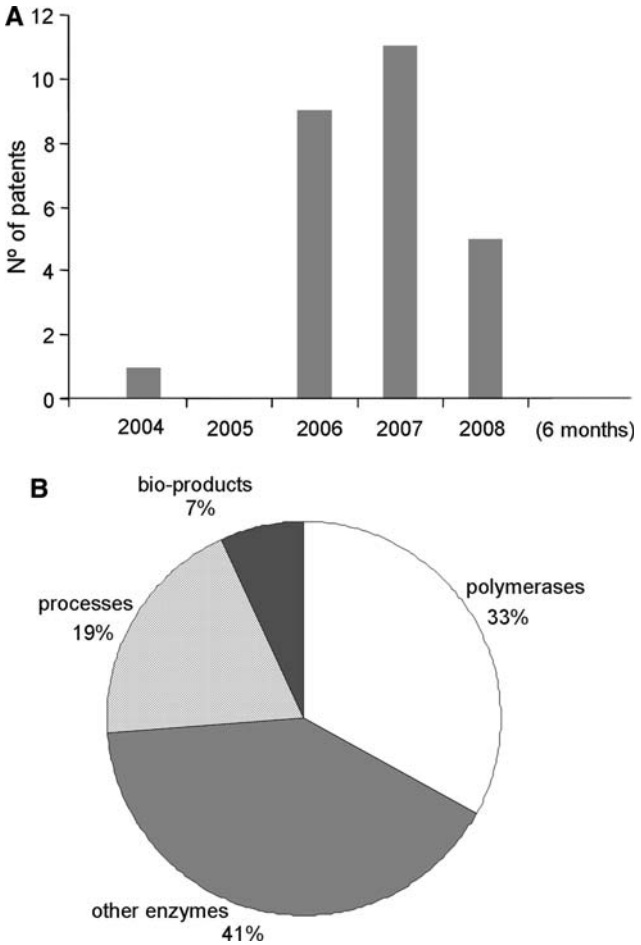

Fig. 3 Patents concerning *Thermus* spp. **a** Number of worldwide *Thermus* related patents per year using the keywords "*Thermus*" and "enzyme". **b** Distribution of worldwide patents of *Thermus* spp. Both graphs derive from data mined from the esp@cenet search utility

Thermozymes other than DNA polymerase account for 41% of all *Thermus* related patents filed in the past 5 years. Among them, a few individual relevant examples can be detailed:

(a) RecA is a multifunctional protein that plays key roles in several cellular processes, such as recombination and DNA repair in bacteria (Lusetti and Cox 2002). Applications of RecA include the protection and deprotection of restriction sites for the controlled digestion of genomes (Ferrin and Camerini-Otero 1991; Szybalski 1997), the capture of small dsDNA fragments (Clontech cloncapture) (Shigemori and Oishi 2004), and the detection of SNPs (Shigemori and Oishi 2005).

(b) Single stranded DNA binding proteins (SSBs) bind and protect single stranded DNA during replication, recombination and repair. The presence of SSBs during DNA replication has been reported to minimize deletion mutagenesis artifacts (Chou 1992) and promotes a faster and more specific DNA amplification independently of the polymerase used (Dabrowski and Kur 1999; Dabrowski et al. 2002; Perales et al. 2003).

(c) Chaperonins from *Thermus* have been reported to both assist in the folding of other proteins (Kohda et al. 2000; Teshima et al. 1998; Witzmann and Bisswanger 1998) and increase the long-term stability of enzymes at lower temperatures.

(d) Galactosidases from *Thermus* sp. have been optimized for their use in the manufacture of lactose-free dairy products, as they could be used simultaneously with the heat treatment to sterilize the product (Pessela et al. 2003, 2007.

(e) Amylomaltase from *Thermus* may be used to modify starches. Although starch is widely used in the food industry as texturizer, native starch must be modified in order to improve its low solubility in cold water, low shear and thermal resistance, poor flavor release properties, and a generally high viscosity (Hansen et al. 2008; Lee et al. 2006, 2008; Park et al. 2007).

(f) Alkaline phosphatase has been described from *T. aquaticus* (Smile et al. 1977), *T. caldophilus* (Park et al. 1999), *T. yunnanensis* (Gong et al. 2005) and *T. thermophilus* (Angelini et al. 2001), and find application in the labeling of primers, the detection of PCR products and as a reporter in promoter probe vectors (Moreno et al., 2003).

(g) DNA ligases have been reported from *T. thermophilus* (Takahashi et al. 1984), *T. scotoductus* (Jónsson et al. 1994) and *T. filiformis* (Kim et al. 1998). Furthermore, NAD-dependent DNA ligase from *Thermus* species display a 1–2 orders of magnitude higher

fidelity than T4 ligase (Luo et al. 1996; Tong et al. 1999). This fact, coupled with its intrinsic thermostability enables the use of Tt ligase in ligase-based technologies, such as ligase chain reaction (Barany 1991) and multi-locus, site-directed mutagenesis (Cline and Hogrefe 2003; Hames et al. 2005).

For a more detailed description of enzymes of potential interest the readers are referred to previous work (Pantazaki et al. 2002).

T. thermophilus as cell factory

The current development of structural genomic programs on thermophilic bacteria and Archaea has revealed that an important number (>40%) of the proteins encoded within a thermophile's genome cannot be expressed or are expressed in an enzymatically inactive form in common mesophilic hosts (Jenney and Adams 2008). For heteromultimeric enzymes, this inability could be due to their requirement for a more or less complex maturation process that could require either specific modification machinery or dedicated chaperones to allow an ordered incorporation of subunits and/or cofactors. Other enzymes, like those that interact with the membrane or have to be secreted through it, could require a relatively specific membrane environment and the adequate processing enzymes. Moreover, there are other cases in which apparently simple enzymes like a Mn-dependent catalase cannot be produced actively in *E. coli* (Hidalgo et al. 2004). For such cases homologous thermophilic cell factories are clearly required.

In this context, several of the aforementioned selection genes have been used to develop expression vectors for *Thermus* spp. Most of them are usually selected by the thermostable kanamycin resistance and include either constitutive or inducible promoters to control the expression of the cloned genes. There are some examples of constitutive and inducible over-expression of proteins in *Thermus* spp that demonstrate the potential of this organisms as cell factory (Hidalgo et al. 2004; Koyama et al. 1990b; Lasa et al. 1992b; Moreno et al. 2005; Park et al. 2004). Constitutive expression has been carried out frequently by using the PslpA promoter (Cava et al. 2007), but other promoters as that of the respiratory complex I (Pnqo) or ribosome proteins have been used. Inducible promoters used include the nitrate reductase (Pnar), the NRC complex (Pnrc) operon promoters or the GroE/L promoters. In some of these cases the protein yield obtained was similar to those obtained by using over-expresion systems of *E. coli* (Moreno et al. 2005).

In most cases, the bacterial strain used is *T. thermophilus* HB27 or a derivative due to its plasmid-free character and to the higher transformation efficiency shown when compared to others (de Grado et al. 1999; Koyama et al. 1986). Such difference could be related to less efficient modification-restriction barrier (de Grado et al. 1999). Whatever the reason is, the impressive transformation yields make this strain an appropriate host for transformation even with gene libraries.

T. thermophilus as host for the thermostabilization of proteins

The use of thermophilic hosts for the selection of thermostable mutants of a given protein has been a promising research field for years. The two articles that pointed out the potential of thermophiles as hosts for such selection method used *Geobacillus* (formerly *Bacillus*) *stearothermophilus* for the thermal selection of a kanamycin nucleotidyl transferase from *S. aureus,* and were published within few months of difference (Liao et al. 1986; Matsumura and Aiba 1985). In both articles, the same two amino acid replacements were identified (Asp80Tyr and Thr130Lys) as responsible for the thermostabilization of the protein. Moreover, one of these mutations (Thr130Lys) was identified a year before in the kanamycin resistance gene encoded by the natural plasmid pTB913 of a thermophilic bacilli (Matsumura et al. 1984), thus supporting that laboratory selection protocols ("directed evolution") could mimic natural selection in the adaptation of proteins to function at higher temperatures.

This type of "functional selection" protocols were subsequently applied to this (Hoseki et al. 1999) and to other thermostable antibiotic resistances in other thermophiles. Thermostable mutants of the Hygromycin B phosphotransferase from *E. coli* were obtained in *Sulfolobus solfataricus* (Cannio et al. 2001), and four years later in *T. thermophilus* (Nakamura et al. 2005). Also, thermostable mutants of the Bleomycin binding protein Shble from *Streptoalloteichus hindustanus* were isolated by selection in *T. thermophilus* (Brouns et al. 2005).

The application of such a functional selection methods to metabolic enzymes is a much more difficult task because it requires the genetic modification of the organism in order to make its growth dependent on the activity of such enzyme under the selection conditions chosen. The first example published was the thermo-adaptation of a 3-isopropylmalate dehydrogenase (LeuB) from *E. coli* by growing on minimal medium a *T. thermophilus* mutant carrying a chimerical *E. coli-Thermus leuB* gene (Tamakoshi et al. 1995). A further refinement of the method was the use a *leuB* mutant of *T. thermophilus* TTY1 as host for the step-by-step thermoselection of mutants of the LEU2 protein from *Saccharomyces cerevisiae* expressed from a plasmid (Tamakoshi et al. 2001). A second example corresponds to the thermo-adaptation of an

alpha-galactosidase from *B. stearothermophilus* KVE39 expressed from a plasmid in a *T. thermophilus* OF1053GD (Δ*agaT*) mutant (Fridjonsson et al. 2002).

Despite the potential of functional selection in a thermophile, it is not always possible to make the organism dependent on a given activity. Moreover, by definition, the method is basically inapplicable to proteins without a known enzymatic activity. To overcome these limitations, the activity independent THR method for the selection of thermostable mutants in *T. thermophilus* has been developed (Chautard et al. 2007). It is based on the "folding interference" principle by which the improper folding of the N-terminal domain of a protein affects to the folding of its C-terminus. Taking advantage of this, a "folding-probe" vector was developed that allowed the constitutive expression in *T. thermophilus* of fusions between a target protein (N-terminal) and the thermostable kanamycin nucleotidyl transferase (C-terminal). The expression of fusions in which a thermosensitive N-terminal protein interferes with the folding of the C-terminal reporter leads to antibiotic sensitivity whereas those mutants with improved folding from a target-focused library provides the cell with selectable levels of antibiotic resistance. This method has been used first to identify amino acid substitutions that improved the folding at high temperatures of small proteins without enzymatic activity such as the human alfa, beta and gamma interferons, and further for the selection of mutants from bacterial enzymes such as a lipase from *Bacillus subtilis* and a 45 kDa formate dehydrogenase from *Pseudomonas* sp. 101 (Chautard et al. 2007).

Concluding remarks

The genus *Thermus* includes a high diversity of thermophilic and extreme thermophilic strains distributed all around the world. *T. thermophilus* isolates easily grow under laboratory conditions and present an extremely efficient natural competence system, two properties that have allowed the development of a wealth in genetic tools and protocols that permit their manipulation to limits so far reached only for mesophilic bacteria "models". On the one hand, this fact and the ability of thermophilic proteins and large protein complexes to crystallize at low temperatures, has led to the use of *Thermus* spp as models for structural biology. On the other hand, it has permitted their use for studies on the physiology of extreme thermophilic bacteria. On applied grounds, *T. thermophilus* can be used as thermophilic factories for the expression of enzymes and even for the selection of thermophilic mutants of mesophilic proteins either through functional selection or through activity independent folding selection protocols.

Acknowledgments This work has been supported by grants of code BIO2007-60245 and S0505/PPQ/0344 from the "Ministerio de Educación y Ciencia" and the "Comunidad Autónoma de Madrid", respectively. A. Hidalgo is supported by a "Ramón y Cajal "contract. An institutional grant from Fundación Ramón Areces to CBMSO is also acknowledged.

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