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ORIGINAL PAPER

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# Heterologous gene expression in *Thermus thermophilus*: β-galactosidase, dibenzothiophene monooxygenase, PNB carboxy esterase, 2-aminobiphenyl-2,3-diol dioxygenase, and chloramphenicol acetyl transferase

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**Abstract** Enzymes from thermophiles are preferred for industrial applications because they generally show improved tolerance to temperature, pressure, solvents, and pH as compared with enzymes from mesophiles. However, nearly all thermostable enzymes used in industrial applications or available commercially are produced as recombinant enzymes in mesophiles, typically *Escherichia coli*. The development of high-temperature bioprocesses, particularly those involving cofactor-requiring enzymes and/or multi-step enzymatic pathways, requires a thermophilic host. The extreme thermophile most amenable to genetic manipulation is *Thermus thermophilus*, but the study of expression of heterologous genes in *T. thermophilus* is in its infancy. While several heterologous genes have previously been expressed in *T. thermophilus* (Fridjonsson et al. in J Bacteriol 184:3385–3391, 2002, Koyama et al. in Appl Environ Microbiol 56:2251–225, 1990, Lasa et al. in J Bacteriol 174:6424–6431, 1992, Mathew et al. in Appl Environ Microbiol 58:421–425, 1992, Takagi et al. in J Ind Microbiol Biotechnol 23:214–217, 1999, Tamakoshi et al. in Extremophiles 5:17–22 2001), the data reported here include the first examples of the functional expression of a gene from an archaeal hyperthermophile (*bglA* from *Pyrococcus woesei*), a cofactor-requiring enzyme (*dszC* from *Rhodococcus erythropolis* IGTS8), and a two-component enzyme (*carBa* and *carBb* from *Sphingomonas* sp. GTIN11). A thermostable derivative of *pnbA* from *Bacillus subtilis* was also expressed, further expanding the list of genes from heterologous hosts that have been expressed in *T. thermophilus*.

**Keywords** *Thermus* · Heterologous · Gene expression

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## Introduction

One of the key opportunities identified in a recent assessment of the future of biotechnology is the study of extremophiles, and particularly thermophiles [1]. Enzymes from thermophiles are not only more resistant to temperature than their counterparts from mesophilic hosts, but they also generally exhibit greater tolerance to changes in pH, exposure to solvents, and exposure to pressure [2–4]. Thus, thermostable enzymes possess qualities that make them more robust and better suited for use in industrial processes. Moreover, bioprocesses performed at high temperatures provide advantages of faster reaction rates, increased solubility of non-gaseous substrates, lower cooling costs, and fewer problems with microbial contamination [5, 6]. Several products derived from thermophiles have already been commercialized, and there is a high level of interest in identifying new enzymes from thermophiles as well as in obtaining thermostable derivatives of enzymes from mesophiles [7–11].

While the genetic manipulation of *Thermus thermophilus* has been possible for several years, there is little data concerning the expression of heterologous genes in this host [1, 12, 13]. The majority of studies of genes from *Thermus* and other species of extreme thermophiles and hyperthermophiles involve the use of *Escherichia coli* as

the host for the cloning and expression of these genes [14–17]. This approach has been quite fruitful, but it also has significant limitations. Some of the most thermotolerant proteins from hyperthermophiles may require chaperones and/or high temperatures in order to fold correctly and yield active enzymes so that potentially interesting enzymes from hyperthermophiles may be missed in genomic libraries prepared and screened in *E. coli* or other mesophilic host species [10, 13, 18]. A thorough characterization of thermotolerant enzymes that require cofactors, that can only be assayed as a part of a pathway, or that require other host-supplied components can only be carried out in thermophilic hosts. Likewise, the thermal adaptation of enzymes from mesophiles would benefit from, and in some cases require the use of a thermophilic host. The study of the expression of heterologous genes in *Thermus* has been limited to the study of individual thermo-stabilized genes, from a limited range of heterologous species: *Staphylococcus aureus* kanamycin (km) nucleotidyl transferase [19], *Bacillus subtilis* subtilisin E [20], *Thermus* sp. T2 α-galactosidase [9, 21], *Clostridium thermocellum celA* [22], and *Saccharomyces cerevisiae* 3-isopropyl malate dehydrogenase [11]. Moreover, these genes are not multi-component enzymes, nor do they require host-supplied cofactors such as NADH and $FMNH_2$. If *T. thermophilus* is to be used in the future development of high-temperature bioprocesses [6], screening of genomic libraries prepared from hyperthermophiles, thermal adaptation of enzymes from mesophiles, and as a host for genetic and biochemical studies of thermotolerant enzymes, it is important to obtain more data concerning the expression of heterologous genes in *T. thermophilus*.

The objective of this study was to examine several heterologous genes from diverse sources to obtain information about gene expression in *T. thermophilus*. Additionally, very few genetic markers are available for use in *Thermus* species [12, 23, 24]. Accordingly, it was also of interest to determine whether alternative genetic markers for use in *T. thermophilus* could be identified. The genes examined here include *bglA*, encoding β-galactosidase from the archaeal culture *Pyrococcus woesei* [14]; *dszC*, encoding the cofactor-requiring dibenzothiophene monooxygenase (DszC) from *Rhodococcus erythropolis* IGTS8 [25]; *pnbA*, encoding a thermostable *p*-nitrobenzyl esterase from *B. subtilis* [26]; *carBa* and *carBb*, encoding 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase from *Sphingomonas* sp. GTIN11 [27]; and the chloramphenicol acetyltransferase (CAT) gene from *S. aureus* [28].

## Materials and methods

### Cultures and plasmids

*Thermus thermophilus* HB27 wild-type was kindly provided by Dr. Glansdorff (Free University of Brussels, Belgium) and was grown in TT rich medium [22]. Plasmid pNB6SF9 [26], containing a thermostable *pnbA* gene, was a gift from Dr. Arnold (California Institute of Technology, Pasadena, Calif., USA). Plasmids pRP9 and pNW26 containing the thermostable CAT gene from *S. aureus* [28, 29] were provided by Dr. Welker (Northwestern University, Evanston, Ill., USA). The plasmid pBR325, which contains the chloramphenicol resistance gene from Tn9, was obtained from culture DSM3865 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *dszC* was obtained from *R. erythropolis* IGTS8 (ATCC 53968) [30]. *carBa* and *carBb* were obtained from *Sphingomonas* sp. GTIN11 [27]. Plasmid pET30 β-gal [31], containing *P. woesei bglA* , was provided by Dr. Synowiecki (Technical University of Gdansk, Poland). pUC18 was purchased from New England Biolabs (Beverly, Mass., USA). *Thermus* expression vectors pTEX1, and pKANPROII-J17 were previously constructed in our laboratory [24, 32], while pTEX4, pMCV1N, and pGTI-phyD were constructed as a part of this work. Plasmid pGEMT-Easy polymerase chain reaction (PCR) cloning vector was purchased from Promega (Madison, Wis., USA). The *Thermus–E.coli* shuttle plasmid pMK18 [33] was a gift from Dr. Berenguer (Universidad Autonoma de Madrid, Spain). The *E. coli* protein expression vector pQE80 was purchased from Qiagen (Hilden, Germany). *E. coli* DH5α competent cells were purchased from Life Technologies (Rockville, Md., USA) and grown using nutrient broth or nutrient agar (Difco, Detroit, Mich., USA). Km was included in liquid or agar medium at 40 and 20 μg/ml in experiments involving either *T. thermophilus* or *E. coli* cultures that were incubated at 55 and 37°C, respectively. Chloramphenicol was employed at 25 and 7 μg/ml in experiments involving either *E. coli* or *T. thermophilus* cultures that were incubated at 37 and 55°C, respectively.

### Chemicals, enzymes, and recombinant techniques

Restriction enzymes were purchased from New England Biolabs. T4 ligase and X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactose) were purchased from Life Technologies. Km, chloramphenicol, $FMNH_2$, NADH, dibenzothiophene (DBT), DBT sulfone ($DBTSO_2$), 2,3-dihydroxybiphenyl, and *p*-nitrophenyl acetate were purchased from Sigma (St. Louis, Mo., USA). Published recombinant techniques [34] were used unless otherwise noted. Transformation of *Thermus*was carried out according to the method of Koyama et al. 1986 [35].

### The DNA polymerase chain reaction

Polymerase chain reaction amplifications were performed with Eppendorf *Taq* DNA polymerase (Eppendorf, Westbury, N.Y., USA), 20 ng of template DNA, 0.25 μM of each primer, 25 μM of dNTP mix (Life Technologies), and 1.5 mM $MgCl_2$ in the buffer recommended by the manufacturer. Amplification was achieved with 25 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 2 min of extension at

72°C, plus an additional extension at 72°C for 7 min with a Perkin Elmer thermal cycler. Amplified DNA was purified with a PCR purification kit (Life Technologies). PCR primers used to amplify the genes used in this study included: $Km^R$ cassette forward 5′-ACACAC TTTAAAAATTCAGATCGCCATCCACCGCCTC-3′, $Km^R$ cassette reverse 5′-ACACACTTTAAATCGT TCAAAATGGTATGCGTTTTGAC- 3′, *dszC* forward 5′-ACACACCATatgAGAATAATGACACTGTCACCT GAAAAGC- 3′, *dszC* reverse 5′-ACACACAGAT CTTCAGGAGGTGAAGCCGGGAATCGGG-3′, *pnbA* forward 5′-ACACACGAATTCCATatgACTCATCA AATAGTAACG- 3′, *pnbA* reverse 5′-ACA CACAAG CTTAGATCTTTATTCTCCTTTTGAAG GGA- 3′, *bglA* forward 5′-ACACACCATatg ATGT TCCCTGA AAAGTTCCTTTGG- 3′, *bglA* reverse 5′-ACA-CACAAGCTT CTTCATCCCCTCAGCAATTCCT CT- 3′, *car Ba* forward 5′-ACACACCATATGGT GGCGGGGGCTTGCCAGTCAGCACA- 3′, *carBb* reverse 5′-ACACACAAGCTTCTAAACGATTGCTA TTCCGCCCA- 3′, cat1 forward 5′-ATCCAA GGATCCT ATTAAAGAGTAGTT-3′, cat1 reverse 5′-ATCCAAC TTAAGTTATAAAAGCCAGTC-3′, cat2 forward 5′-ATCCAACTGCAGGG GAATGAGAATA AATGAACTTTAATAA- 3′, cat2 reverse 5′-ATCCA AGGATCCTTATAAAAGCCAGT C-3′, cat3 forward 5′-ATCCAAAGATCTTATTAAA GAGTAGTT-3′, cat3 reverse 5′-ATCCAAAGATCTTTATAAAAGCCAGTC 3′, cat4 forward 5′-ACAACAAGATCTAGATCGCCAT CCAC-3′, and cat4 reverse 5′-ACAACAAGATCTT TATAAAAGCCAGT-3′. The PCR primers listed above are based on the DNA sequences of GenBank accession numbers L37363 (*R. erythropolis dszC*), U06089 (*B. subtilis pnbA*), AF043283 (*P. woesei bglA*), AF442494 (*Sphingomonas* sp. GTIN11 *carBa* and *carBb*), and NC002013 (*S. aureus*, CAT gene). The Km resistance ($Km^R$) cassette was amplified from pTEX1 [32]. Underlined sequences were chosen to facilitate cloning using *Dra*I for the $Km^R$ cassette, 5′ *Nde*I and a 3′ *Bgl*II site for *dszC*, 5′ *Nde*I and 3′ *Hin*dIII for *pnbA*, *bglA*, and for *carBa*/*carBb*, 5′ *Bam*HI and ′ *Afl*II for cat1, 5′ *Pst*I and 3′ *Bam*HI for cat2, 5′*Bgl*II and 3′ *Bgl*II for cat3, and 5′ *Bgl*II and 3′ *Bgl*II for cat4 and subsequent ligation into the corresponding restriction endonuclease cleavage sites of the pQE80, pTEX1, pTEX4, pMCV1N, pKANPROII-J17, and pGTE- *phyD* vectors employed in gene expression experiments.

## Determination of β-galactosidase specific activities

Cell-free extracts were prepared for the β-galactosidase activity tests by growing *T. thermophilus* HB27 cultures in TT media at 55°C until mid-to late-exponential-phase growth was achieved. Cell pellets were harvested by centrifugation at 10,000 *g* for 10 min at room temperature, washed once with 0.1 M potassium phosphate (pH 7.0) buffer and resuspended in the same buffer. The cell suspension was disrupted by sonication using a Branson 350 sonifier (Branson Sonifer, Danbury, Conn., USA) equipped with a microtip. The cell-free extracts were centrifuged at 10,000 *g* for 15 min at room temperature to remove cellular debris. β-Galactosidase activity was determined using a spectrophotometric assay at 420 nm according to the manufacturer's protocol (Promega). The assay was done by incubating the reaction mixture (150 µl assay buffer and 150 µl cell lysate) in a microfuge tube at 37°C for 30 min. One unit of β-galactosidase activity was defined as 1 nmol o-nitrophenol liberated from o-nitrophenol-β-D-galactoside/$min^{-1}$ at 37°C. Specific activity was expressed as β-galactosidase units/mg protein. Protein content was determined using the bicinchoninic acid protein assay system (Pierce Chemical, Rockford, Ill., USA). Bovine serum albumin was used as a protein standard. β-Galactosidase values are the averages of at least four experiments, with a variation of no more than 10% from the mean.

## *dszC* expression in *T. thermophilus* HB27

In growing cell experiments *T. thermophilus* HB27 and *T. thermophilus* HB27/pTEX1- *dszC* were cultivated in 250-ml Erlenmeyer flasks containing 50 ml ATCC medium 697 [22] (TT medium) supplemented with 28 mg DBT. Flasks were incubated on a shaking platform at 55°C for 16 h until the culture reached an optical density of 1.2 at 600 nm. DBT was added in excess of its aqueous solubility, which is 0.0061 mM [36], to maintain saturating concentrations. The overnight cultures were acidified with 6 N HCl to a pH 3.0, extracted three times with ethyl acetate, and then the extracts were concentrated and analyzed by HPLC for DBT and $DBTSO_2$ [25]. Briefly, a Waters liquid chromatograph equipped with a photodiode array detector and fitted with a WATERS SYMMETRY C18 column was used. The mobile phase was acetonitrile and phosphate buffer (pH 6.0, 10 mM) mixed 1:1. Crude cell lysates were prepared by sonication [24, 32] from *T. thermophilus* and from *T. thermophilus* HB27/pTEX1- *dszC* grown at 55°C. These cell lysates were assayed for DszC activity by measuring the amounts of $DBTSO_2$ formed at 37°C in 1 h. The standard reaction mixture, in a microcentrifuge tube, contained 100 mM potassium phosphate buffer (pH7.0), 200 mM DBT, 4 mM $NADH_2$, 10 µM $FMNH_2$, and a suitable amount of crude cell lysate in a total volume of 750 µl. Reactions were terminated by the addition of 750 µl acetonitrile and thorough mixing. DBT and $DBTSO_2$ were quantified using HPLC [25].

## Determination of *p*-nitrobenzyl esterase activity

Shake flasks containing TT media with Km were inoculated with an overnight culture (4% v/v) of *T. thermophilus* or *E. coli* and grown at 55 or 37°C, respectively, to mid-exponential phase. Samples were washed, resuspended in 10 mM Na-phosphate, pH 7.0

(1 g cells/4 ml buffer) and disrupted by sonication. After removal of cell debris by centrifugation, the extracts were assayed for total protein, and for activity with *p*-nitrophenyl acetate. Cell extract (1–20 µl) was added to 1 ml enzyme assay buffer consisting of 400 µl of 2 mM *p*-nitrophenyl acetate (in 1% acetonitrile)/600 µl of 167 mM Tris-HCl, pH 7.0. Formation of *p*-nitrophenol was measured spectrophometrically at 410 nm ($\eta$ = $15\times10^3$ M/cm) [26]. One unit of esterase activity was defined as the amount of enzyme that released 1 µmol *p*-nitrophenol/min.

### Assay for FMN oxidoreductase

The FMN oxidoreductase specific activity was quantified by a spectrophotometric assay that monitored NADH absorbance at 340 nm [37]. One unit of FMN oxidoreductase enzyme activity was defined as the amount needed to convert 1 µmol NADH to NAD in 1 min.

### Determination of 2,3-dihydroxybiphenyl dioxygenase activity

*Escherichia coli* DH5α colonies with recombinant plasmids were screened for 2,3-dihydroxybiphenyl dioxygenase activity by spraying the colonies on plates with a 1-g/l solution of 2,3-dihydroxybiphenyl in ether and visually observing the formation of a yellow metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) [38].

## Results

### Expression of *P. woesei bglA* in *T. thermophilus* HB27

To examine the expression of *P. woesei bglA* in *T. thermophilus*, a derivative that contained a deletion of the native β-galactosidase gene, *T. thermophilus* PPKU [39], was used. A *Thermus* expression vector capable of also replicating in *E. coli*, pTEX4 was formed by inserting the ampicillin resistance gene from pUC18 into the *Sph*I site of pMK18. pTEX4 was subsequently modified by the addition of the Psyn promoter, which allows for the expression of cloned genes in both *T. thermophilus* and *E. coli* (data not shown), *bglA*, encoding a thermostable β-galactosidase from *P. woesei*, and a $Km^R$ resistance gene to yield pTEX4-Pw *bglA* as shown in Fig. 1. The level of β-galactosidase in *T. thermophilus* PPKU was 0.15 ± 0.01 units/mg protein, while *T. thermophilus* PPKU/pTEX4-Pw *bglA* yielded 0.8 ± 0.02 units/mg protein, or more than a fivefold increase.

### Expression of *R. erythropolis dszC* in *T. thermophilus* HB27

Dibenzothiophene monooxygenase requires NADH, $FMNH_2$, and oxygen to catalyze the conversion of DBT to $DBTSO_2$ with the aid of NADH-FMN oxidoreductase [25, 37]. Prior to testing for *dszC* expression in *Thermus*, the intrinsic NADH-FMN oxidoreductase or flavin reductase activity of *T. thermophilus* HB27 and of *E. coli* DH5α were quantified; these were 0.31 and 1.26 units/mg protein, respectively, as compared to 5 units/mg protein reported for *R. erythropolis* IGTS8 [40].

*dszC* was amplified from *R. erythropolis* IGTS8 genomic DNA and cloned into the *Nde*I/ *Bam*HI site of *Thermus* sp. expression vector pTEX1 [24, 32] to create plasmid pTEX1-*dszC*. RT-PCR was used to amplify *dszC* transcripts in total RNA isolated from *T. thermophilus* HB27/pTEX1- *dszC* cells grown at 55°C and *dszC* cDNA was detected by agarose gel electrophoresis (data not shown). The presence of a functional DszC enzyme

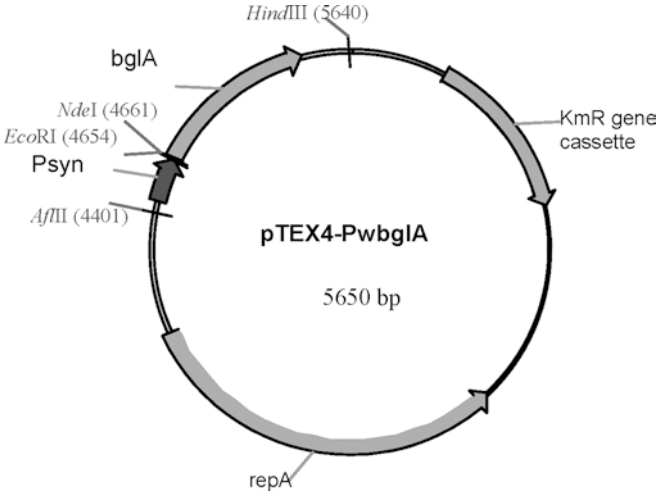


**Fig. 1** Construction of *Thermus*/*E. coli* Expression Vectors pTEX4-Pwb *glA*, pTEX4-*pnbA*,and pTEX4-*carB*. The *Sph*I- *Sph*I fragment of pMK18 was inserted into the largest *AflIII*/*NdeI* fragment of pUC18 to obtain pTEX4. The promoter fragment Psyn consists of the Pslp promoter region (*underlined*) [48] and the ribosomal binding site (*italicized*) sequence derived from the P31 *Thermus* promoter [20] (GAGTCGAGTATAA CAGAAACCT-TAAGGCCCGACCGCTTGACAAGGGGCGCGTG AGGTT TT TTACGATAGCGATA GCGCC GG*GGGGAA TGAGAATA*). The distance between the ribosome binding site (RBS) and the start codon of genes expressed by the Psyn promoter in this study was 10 bp. Restriction sites were added into the synthetic promoter PCR products to give 5′ *Afl*II and 3′ *Eco*RI *Nde*I sites and facilitate cloning into *Afl*II/ *Nde*I-cleaved pTEX4 to yield pTEX4-Psyn, which replaced the *slpA* promoter in pTEX4 with the Psyn promoter. *P. woesei bglA* was PCR-amplified and the product was cloned into *Eco*RI-*Hind*IIII-digested pTEX4-Psyn to create pTEX4-Psyn- *bglA*. A $Km^R$ resistance cassette was amplified using plasmid pTEX1 as the template and using the PCR primers "$Km^R$ cassette" to give 5′ and 3′ *Dra*I sites. The cassette contains a strong constitutive *Thermus* promoter (J17) upstream of the thermostable Km nucleotidyltransferase gene. To prevent transcription read-through from the Psyn promoter, a transcriptional termination sequence was cloned upstream of the J17 promoter [24]. The $Km^R$ cassette was digested with *Dra*I and ligated into the *Dra*I-digested pTEX4 Psyn- *bglA*, resulting in pTEX4- *bglA*. Vectors pTEX4-*pnbA*, and pTEX4- *carB* were constructed in a fashion analogous to pTEX4- *bglA* by substituting DNA fragments containing *B. subtilis pnbA* or *Sphingomonas* sp. GTIN11 *carBa* and *carBb* for the *P. woesei bglA* gene fragment

in *Thermus* was confirmed by the accumulation of $DBTSO_2$ in cultures of *T. thermophilus*HB27/pTEX1-*dszC* growing at 55°C. $DBTSO_2$ was absent in the *T. thermophilus* HB27 controlflask. DszC activity was also observed in crude cell lysates [32] from cultures grown at grown at 55°C, as 23.6 μM $DBTSO_2$ was detected by HPLC in reactions employing crude cell lysates derived from *T. thermophilus*HB27/pTEX1- *dszC* while no $DBTSO_2$ was detected in reactions employing *T. thermophilus* HB27 lysates.

## Expression of *pnbA* from *B. subtilis* in *T. thermophilus* HB27

*B. subtilis pnbA* was amplified and cloned so as to replace *bglA* in pTEX4-Pw *bglA* to yield pTEX4 -*pnbA* in a manner analogous to the construction of pTEX4-Pw *bglA* as shown in Fig. 1. *E. coli* DH5α and *T. thermophilus* HB27 cells were transformed with pTEX4-*pnbA*. The results shown in Table 1 clearly demonstrate the expression of *pnbA* in *E. coli* and *T. thermophilus* HB27 despite low levels of background interferences. In the presence of pTEX4-*pnbA*, *p*-nitrobenzyl esterase activity was increased tenfold in both *E. coli* and *Thermus*. Maximal esterase activity was observed in *T. thermophilus* HB27/pTEX4-*pnbA* cells grown at 52–60°C as shown in Fig. 2.

## Expression of *Sphingomonas* sp. GTIN11 *carBa* and *carBb* in *T. thermophilus* HB27

*carBa* and *carBb* were amplified from *Sphingomonas* sp. GTIN11 genomic DNA and cloned so as to replace *bglA* in pTEX4-Pw *bglA* to yield pTEX4-*carB* in a manner analogous to the construction of pTEX4-Pw *bglA* as shown in Fig. 1. *T. thermophilus* HB27/pTEX4-*carB* was positive in the 2,3-dihydroxybiphenyl dioxygenase assay, whereas *T. thermophilus* HB27 was not.

## Expression of the *S. aureus* chloramphenicol acetyl transferase gene in *T. thermophilus* HB27

The CAT from *S. aureus* has been shown to be thermally stable at temperatures up to 60°C in *B. stearothermophilus* containing pRP9 and related plasmids [29]. To investigate the expression of the CAT gene in *T. thermophilus* HB27, plasmid vector pMCV1N, which contains the CAT gene from pRP9 plus replication genes for *T. thermophilus*, *E. coli*, and *B. stearothermophilus*, was constructed (data not shown). While pMCV1N yielded $Cm^R$ transformants in *E. coli*, no $Cm^R$ transformants of *T. thermophilus* HB27 were obtained.

This CAT gene was also cloned in several other vectors in an effort to obtain $Cm^R$ in *T. thermophilus* HB27. The CAT gene, including its promoter, was amplified from pRP9 using PCR primers cat1, and ligated into the *Thermus*/*E. coli* vector pKANPROII-J17 [24]. The resulting construct, pKII-CAT, yielded $Cm^R$ and $Km^R$ transformants in *E. coli*, but only $Km^R$ transformants in *T. thermophilus*. *T. thermophilus* HB27/pKII-CAT $Km^R$ transformants failed to grow when streaked onto plates containing Cm. PCR primers cat2 were used to amplify only the coding sequences of the CAT gene. The DNA fragment produced from the amplification of pRP9 with primers cat2 was ligated into pKANPROII-J17 in such a way that the $Km^R$ gene was replaced by the CAT gene, yielding pKII-J17-CAT (Fig. 3), in which the CAT gene was immediately downstream from a promoter, J17, and the ribosome binding site (RBS), which functions in both *T. thermophilus* and *E. coli* [24]. $Cm^R$ transformants of *E. coli*, but not of *T. thermophilus*, were obtained with pKII-J17-CAT.

The PCR primer pairs cat3 and cat4 were used to amplify the CAT gene from pRP9 to yield CAT gene fragments that contained its native promoter or the *Thermus* J17 promoter, respectively, as shown in Fig. 3. These CAT gene fragments were ligated into the proprietary *Thermus* integrative vector pGTI- *phyD* to yield pGTI- *phyD*::CAT and pGTI- *phyD*::J17-CAT, respectively, as shown in Fig. 3. The integrative vector pGTI-*phyD* lacks the ability to replicate in *T. thermophilus*, but contains a portion of *T. thermophilus phyD*, which allows it to integrate into the chromosome by homologous

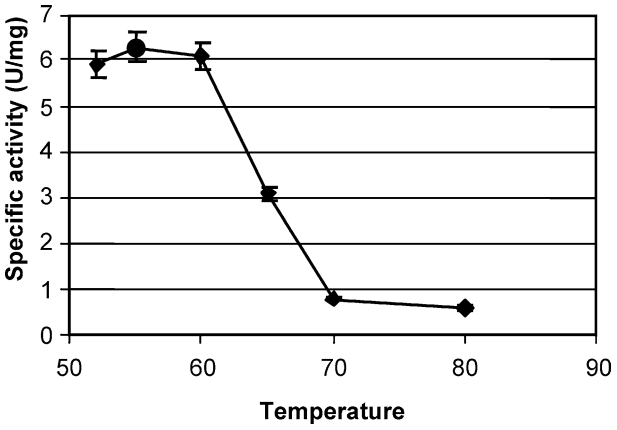


**Fig. 2** Effect of temperature on esterase activity in growing *T. thermophilus* HB27/pTEX4-*pnbA* cells. Cells were grown at 52–80°C and crude cell extracts were prepared for the determination of esterase enzyme activities at 60°C using 167 mM *p*-nitrophenyl acetate in Tris-HCl, pH 7.0. Measurements are the mean ± (*bars* standard deviations) for at least three independent measurements

**Table 1** Para nitrobenzyl esterase activities in *Escherichia coli* and *Thermus thermophilus* HB27. *E. coli* cells were grown in LB at 37°C and *Thermus* cells were grown TT medium at 60°C; all assays were performed at 60°C. Values recorded are averages, ± standard deviation (SD), of three replicate samples

| Culture and condition | Esterase activity (units/mg) ± SD |
|---|---|
| *E. coli* DH5α | 1.0 ± 0.14 |
| DH5α/pTEX4-*pnbA* | 10.3 ± 0.32 |
| *T. thermophilus* HB27 | 0.6 ± 0.18 |
| HB27/pTEX4-*pnbA* | 6.3 ± 0.43 |

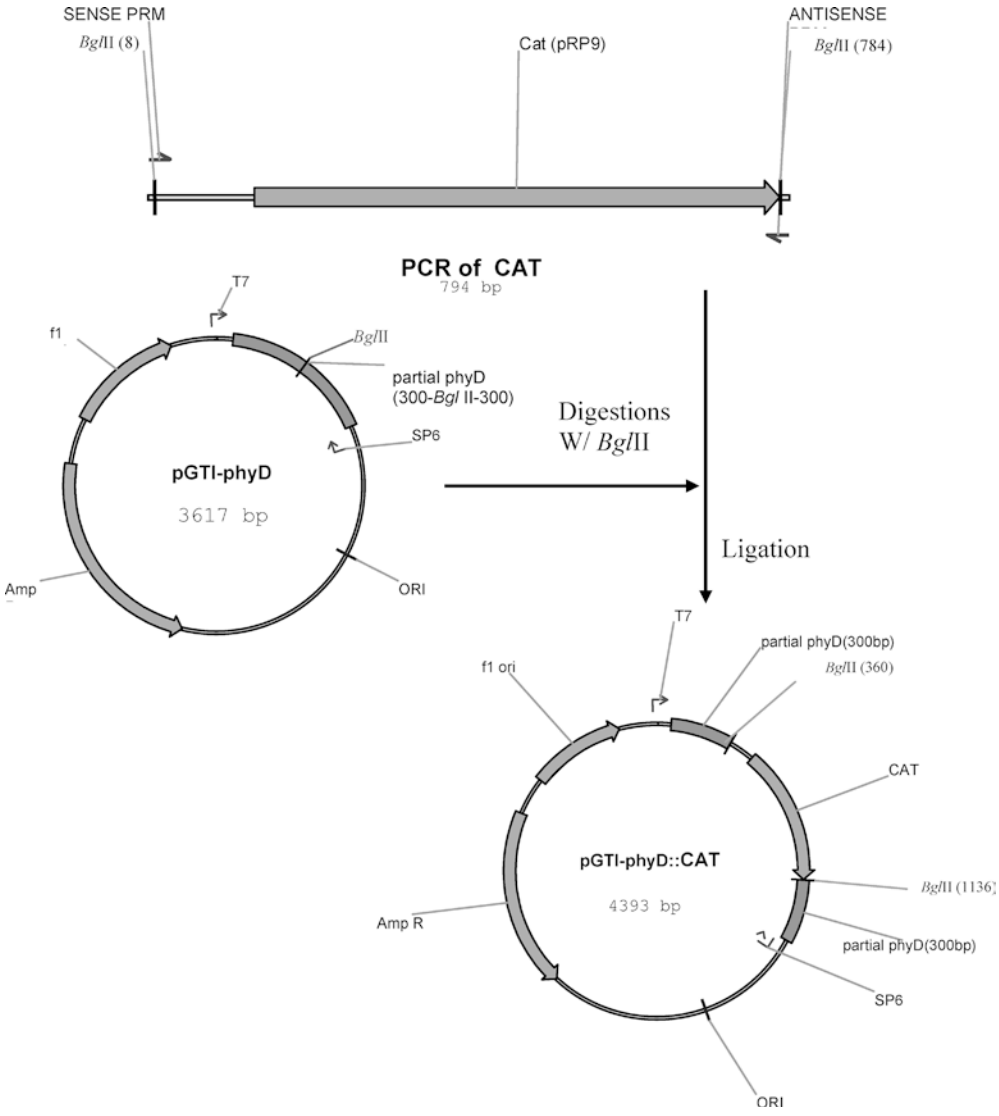


**Fig. 3** Construction of *Thermus* integration vectors pGTI-*phyD*::CAT and pGTI-*phyD*::J17-CAT. A DNA fragment consisting of about 600 bp of *T. thermophilus phyD* was cloned into pGEMT-Easy to create pGTI-*phyD*. The CAT gene and its promoter were amplified from pRP9 using primers cat3 to yield a DNA fragment containing 5′ and 3′ *Bgl*II sites. When this *Bgl*II fragment was cloned into the *Bgl*II site of pGTI-*phyD* it yielded a plasmid, pGTI-*phyD*::CAT, that contained the CAT gene flanked by 300 bp of DNA homologous to *T. thermophilus phyD* on a vector capable of replicating only in *E. coli*. A similar vector, pGTI-phyD::J17-CAT, was produced by using PCR primers cat4 to amplify the J17 promoter [24] plus the CAT gene from pKII-J17-CAT, which was ligated to pGTI- *phyD* linearized with *Bgl*II

recombination. The integration of pGTI- *phyD* into the *T. thermophilus* chromosome resulted in the insertional inactivation of phytoene dehydrogenase, an enzyme in the carotene synthesis pathway [41], allowing transformants to be detected due to a lack of pigmentation. Successful transformation and integration of both pGTI- *phyD*::CAT and pGTI- *phyD*::J17-CAT into *T. thermophilus* HB27 was achieved, but no $Cm^R$ colonies were obtained.

Additionally, error-prone PCR [42] was employed using primer pairs cat1 and cat2 to produce libraries of mutagenized derivatives of the CAT gene that were subsequently cloned to produce plasmids analogous to pKII-CAT and pKII-J17-CAT. However, no $Cm^R$ transformants of *T. thermophilus* were obtained.

Constructs similar to pKII-CAT and pKII-J17-CAT were made using the chloramphenicol resistance gene derived from Tn9 [41] but no expression of chloramphenicol resistance in *T. thermophilus* was obtained (data not shown). During efforts to obtain $Cm^R$ derivatives of *T. thermophilus* using the chloramphenicol resistance genes of Tn9 or pRP9, *T. thermophilus* cultures that showed reproducible resistance to chloramphenicol on agar plates at concentrations up to 10 $\mu$/g ml were isolated; however; plasmid-associated $Cm^R$ could not be demonstrated for these cultures and their $Cm^R$ was presumed to have resulted from unidentified host mutations.

## Discussion

There are numerous advantages to the use of thermophiles, such as obtaining thermostable enzymes and the development of high-temperature bioprocesses. Since many microbial species cannot yet be cultivated in the laboratory[43], obtaining thermostable enzymes from extreme and hyperthermophiles could benefit from the use of a thermophilic host for screening of genomic libraries prepared from environmental samples. A thermophilic host provides chaperones and/or other factors that, in addition to high temperatures, may be needed

for some thermotolerant enzymes to fold correctly and to assume an active conformation so they can be detected in screening assays. It is common to derive thermostable mutants of enzymes by screening libraries of mutagenized genes in *E. coli* [20, 26]. However, for applications in which selection rather than screening is possible, it would be far more convenient to utilize a thermophilic host to directly obtain thermostabilized derivatives of genes of interest, or to select thermostable enzymes present in genomic libraries prepared from samples obtained from high-temperature environments [44]. The feasibility of this approach is indicated by the results reported here, which include the expression of *bglA* from the archaeal hyperthermophile *P. woesei*. This gene has a GC content of 43% as compared with 69.3% for chromosomal DNA from *T. thermophilus*. Nevertheless, functional $\beta$-galactosidase was produced at levels fivefold higher than background. This is the first report of the expression of a gene from a hyperthermophilic archaeal species in *T. thermophilus*. These preliminary studies did not include optimization of gene expression by adjusting codon usage patterns, transcriptional, or translational signals, yet functional expression of *P. woesei bglA* was obtained in *T. thermophilus*. This is particularly noteworthy because many hyperthermophiles are archaeal species and may be preferred sources for future thermotolerant enzymes [1]. Since genetic vectors for the cloning and expression of genes in archaeal hyperthermophiles are generally not available, the use of *T. thermophilus* as a host in gene expression experiments could provide several practical advantages.

*T. thermophilus* has the best developed genetic tools of all thermophiles, yet the range of heterologous genes expressed in the bacterium has been rather modest thus far. The results reported here demonstrate that several additional heterologous genes, including a cofactor-requiring enzyme (DszC encoded by *dszC* from *R. erythropolis* IGTS8) and a two-component enzyme (2-aminobiphenyl 2,3-diol 1,2-dioxygenase encoded by *carBa* and *carBb* of *Sphingomonas* sp. GTIN11) can be expressed in *T. thermophilus*. DszC requires NADH, $FMNH_2$, and oxygen to catalyze the transformation of DBT to $DBTO_2$ and is therefore highly dependent upon host factors, which may also include those neeeded for substrate transport [25, 45]. As a two-component/subunit enzyme CarB is likewise dependent upon host factors to allow the production, folding and assembly of active enzyme [46]. Therefore, the functional expression of both DszC and CarB is promising for the eventual development of high temperature bioprocesses in *T. thermophilus*.

It is also noteworthy that neither of these enzymes is thought to be thermostable, as both originate from mesophiles and have not been subjected to thermostabilization. The temperature optimum for DszC was 30°C in *R. erythropolis*, but 50°C in *Mycobacterium phlei*, corresponding to the optimum growth temperatures for these two cultures [45]. In this study, DszC activity was detected in *T. thermophilus* at 55°C, further illustrating that properties of the host could influence the temperature at which activity of various enzymes can be detected. The 2'-aminobiphenol-2,3-diol 1,2-dioxygenase enzyme encoded by *carBa* and *carBb* has a temperature optimum of 37°C in *Sphingomonas* sp. GTIN11 [27], yet displayed detectable activity at 55°C in *T. thermophilus*. This further demonstrates that it may be possible to functionally express a variety of genes from mesophiles in *T. thermophilus* and obtain sufficient activity to detect their presence and allow subsequent selection for thermostable derivatives. The level of DszC activity in *T. thermophilus* was rather low, but it is likely that activity could be greatly increased with further experimentation. The level of FMN oxidoreductase activity in *T. thermophilus* was 15-fold less than the specific activity in *R. erythropolis*. The thermolability of DszC could be improved, and adjusting codon usage within *dszC* versus typical *Thermus* genes are both possible means of improving the activity of DszC in *T. thermophilus*.

In this study, the thermostabilized *B. subtilis p*-nitrobenzyl esterase (*pnbA*) gene was cloned into the pGTI-TEX4- *pnbA* expression vector and transformed into both *E. coli* and *T. thermophilus*. Esterase activity was somewhat higher in *E. coli* (10.3 units/mg) than in *T. thermophilus* (6.3 units/mg) but the magnitude of increase in esterase activity versus background levels in each strain was essentially the same: a tenfold increase was observed. The thermostability of PnbA activity in *T. thermophilus* was identical to that reported for the purified enzyme [26], further expanding the list of heterologous genes successfully expressed in *T. thermophilus*.

It is worth noting that not all heterologous genes, even genes encoding thermostable proteins, can be readily expressed in *T. thermophilus*. We tried unsuccessfully to express the CAT genes from pRP9 and Tn9 in *T. thermophilus* HB27. The CAT gene encoded by pRP9 has been the drug resistance gene of choice for the construction of genetic vectors for use in *B. stearothermophilus*, as this enzyme shows good stability at temperatures of at least 60°C [29]. Multiple attempts employing different CAT gene fragments, different *Thermus* promoters, plasmid and integrative constructs all proved unsuccessful. The reason(s) for the lack of functional expression of the pRP9 and Tn9 CAT genes in *T. thermophilus* is unknown, and serves to highlight the need for further research concerning gene expression in *T. thermophilus*. It would be instructive to quantify CAT gene transcription, and the stability of CAT mRNA in various *Thermus* expression vectors.

This study demonstrated the use of several other genes that may be used as convenient genetic markers in *T. thermophilus*. The $\beta$-galactosidase encoded by *bglA* from *P. woesei*, the 2'-aminobiphenyl 2,3-diol 1,2-dioxygenase encoded by *carBa* and *carBb* from *Sphingomonas* sp. GTIN11, and the *p*-nitrobenzyl esterase encoded by *pnbA* from *B. subtilis* all have convenient enzymatic assays and can be used in chromogenic

reactions to allow the expression of these genes to be detected on agar plates [14, 26, 27]. Additionally, this work included the construction and use of vectors employing the *T. thermophilus phyD* . Since the product of *phyD* is essential for the synthesis of carotenoid pigments in *T. thermophilus* [47], cultures in which *phyD* has been disrupted by the integration of pGTI- *phyD* vectors can be detected because of a loss of pigmentation. These genes, and the recently described vectors employing *T. thermophilus mdh* [24], make useful additions to the short list of genetic markers that can conveniently be used in genetic studies of *Thermus* species.

The results reported here demonstrate that heterologous genes from a variety of diverse species can be functionally expressed in *T. thermophilus*. Even cofactor-requiring and multi-component enzymes from heterologous species were successfully expressed in *T. thermophilus*. The ease and high efficiency with which *T. thermophilus* can be transformed and the availability of cloning and expression vectors make it appropriate to consider using *T. thermophilus* rather than *E. coli* for future genetic and biochemical studies that involve thermostable enzymes.

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