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Thermoadaptation-directed evolution of chloramphenicol acetyltransferase in an error-prone thermophile using improved procedures

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Abstract Enhancing the thermostability of thermolabile enzymes extends their practical utility. We previously demonstrated that an error-prone thermophile derived from *Geobacillus kaustophilus* HTA426 can generate mutant genes encoding enzyme variants that are more thermostable than the parent enzyme. Here, we used this approach, termed as thermoadaptation-directed enzyme evolution, to increase the thermostability of the chloramphenicol acetyltransferase (CAT) of *Staphylococcus aureus* and successfully generated a CAT variant with an A138T replacement (CAT^{A138T}). This variant was heterologously produced, and its enzymatic properties were compared with those of the wild type. We found that CAT^{A138T} had substantially higher thermostability than CAT but had comparable activities, showing that the A138T replacement enhanced protein thermostability without affecting the catalytic activity. Because variants CAT^{A138S} and CAT^{A138V}, which were generated via in vitro site-directed mutagenesis, were more thermostable than CAT, the thermostability enhancement resulting from the A138T replacement can be attributed to both the presence of a hydroxyl group and the bulk of the threonine side chain. CAT^{A138T} conferred chloramphenicol resistance to *G. kaustophilus* cells at high temperature more efficiently than CAT. Therefore, the gene encoding CAT^{A138T} may be useful as a genetic marker in *Geobacillus* spp. Notably, CAT^{A138T} generation was achieved only by implementing improved procedures (plasmid-based mutations on solid media); previous procedures (chromosome-based mutations in liquid media) were unsuccessful. This result suggests that this improved procedure is crucial for successful thermoadaptation-directed evolution in certain cases and increases the opportunities for generating thermostable enzymes.

Keywords *Geobacillus kaustophilus* · Chloramphenicol resistance · Chloramphenicol acetyltransferase · Thermoadaptation-directed evolution · Error-prone thermophile

Introduction

Although enzymes have been used as catalysts in a variety of commercial applications (e.g., drugs, medical diagnostics, pulp bleaching, starch processing, biosensors, and detergents), many enzymes lack practical utility despite their useful catalytic activities, primarily because their thermolability hinders their prolonged use as catalysts and long-term storage at ordinary temperatures. In particular, although enzymes identified in mesophiles have efficient catalytic activities at moderate temperatures, they are thermolabile and readily inactivated. In contrast, most enzymes isolated from extreme thermophiles are thermostable but are inefficient catalysts at moderate temperatures. Therefore, enzymes from both mesophiles and extreme thermophiles are often impractical for applications that require efficient enzymatic activity at room temperature (e.g., medical diagnostics and biosensors). Protein engineering is a promising approach to overcome this challenge. Useful catalysts can be obtained by enhancing the thermostability of thermolabile enzymes while maintaining efficient catalytic activities at moderate temperatures.

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There are two main approaches for enhancing enzyme thermostability. In one approach, in silico rational design based upon structural information about target enzymes is used to predict variants with enhanced thermostability (Huang et al. 2012; Fang et al. 2014). The predicted variants are then enzymatically characterized in vitro to confirm their actual thermostability. This approach is effective for narrowing the list of candidates for enzymatic characterization but is inapplicable without structural information. The other approach uses random mutagenesis in vitro to construct an enzyme variant library followed by screening this library to identify thermostable variants. This approach often requires enzymatic characterization of numerous variants in vitro (Asako et al. 2008; Ben Mabrouk et al. 2013; Stephens et al. 2014). However, in several cases, thermophiles have been used as library hosts to facilitate the screening process because thermostable enzymes are distinguishable from thermolabile enzymes during the growth of thermophiles at high temperatures when target enzyme activities are detected in vivo (Matsumura and Aiba 1985; Liao et al. 1986; Hoseki et al. 1999; Tamakoshi et al. 2001; Fridjonsson et al. 2002; Brouns et al. 2005; Nakamura et al. 2005). Although not a main approach, some thermostable enzymes have also been generated via spontaneous mutations in thermophiles, a process termed as thermoadaptation-directed enzyme evolution. Examples include the generation of thermostable variants of hygromycin B phosphotransferase (Nakamura et al. 2005), α-galactosidase (Fridjonsson et al. 2002), 3-isopropylmalate dehydrogenase (Tamakoshi et al. 2001), and kanamycin nucleotidyltransferase (Liao et al. 1986).

Geobacillus kaustophilus HTA426 (JCM 12893) is an aerobic, Gram-positive, *Bacillus*-related thermophile for which genetic tools have been established (Suzuki et al. 2012; Suzuki and Yoshida 2012; Suzuki et al. 2013a, b) and a whole genome sequence is available (Takami et al. 2004). In a previous study (Suzuki et al. 2015), we constructed an error-prone thermophile, MK480, from *G. kaustophilus* HTA426 and then used this strain to examine the thermoadaptation-directed evolution of orotidine 5′-phosphate decarboxylase of *Bacillus subtilis* 168. This procedure successfully generated two mutant genes encoding thermostable variants, which suggested that this approach may efficiently generate thermostable variants from thermolabile enzymes. However, a single practical example is not sufficient to establish the versatility of this approach.

Therefore, this study examined the thermoadaptation-directed evolution of the chloramphenicol acetyltransferase (CAT) of *Staphylococcus aureus*. The gene encoding this enzyme is a proven but inefficient genetic marker for *Geobacillus* spp. presumably because of insufficient enzyme stability at high temperatures.

Materials and methods

Bacterial strains, media, and primers

G. kaustophilus MK242 and MK480 (Table 1) were previously constructed (Suzuki et al. 2015) and used as genetically stable and error-prone strains, respectively. These strains were grown at 60 °C in Luria–Bertani (LB) medium. However, cells carrying plasmids derived from pGKE70 or pGKE75 (see below) were cultured in LB medium supplemented with 5 mg l^{-1} kanamycin (LK5). *Escherichia coli* DH5α was used for DNA manipulations. *E. coli* BL21-CodonPlus(DE3)-RIPL (Novagen, Darmstadt, Germany) was used to produce recombinant proteins. *E. coli* strains were grown at 37 °C in LB medium. Ampicillin (50 mg l^{-1}) and chloramphenicol (Cm; 50 mg l^{-1}) were added as necessary. The primer sequences are summarized in Table 2.

Construction of plasmids

The plasmid pGKE70, which is capable of autonomous replication in *E. coli* and not in *G. kaustophilus*, was previously constructed (Suzuki et al. 2015). This plasmid was used to integrate genes into the *trpE* locus (*GK2204*) of *G. kaustophilus* and to induce gene expression in *G. kaustophilus* under the control of the P_{gk704} promoter (Suzuki et al. 2013b). The plasmid pGKE75 (Fig. 1) was constructed from pGKE73 (Suzuki et al. 2013a) by removing the *Sph*I site in the pBST1 replication region using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA) and primers sphU and sphD. This plasmid, which is capable of autonomous replication in both *E. coli* and

Table 1 *G. kaustophilus* strains used in this study

Strain	Genotype
MK242	Derivative of the wild-type strain HTA426 (JCM 12893); Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) *GK0707*::P_{gk704}-*bgaB*
MK480	Derivative of the MK242 strain; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) *GK0707*::P_{gk704}-*bgaB* Δ(*mutS*-*mutL*) Δ*mutY* Δ*ung* Δ*mfd*

G. kaustophilus MK242 and MK480 lack genes related to pyrimidine biosynthesis (*pyrF* and *pyrR*) and DNA restriction–modification ($hsdM_1S_1R_1$, $mcrB_1$, $mcrB_2$, $hsdM_2S_2R_2$, and *mrr*). The MK480 strain further lacks genes related to DNA repair (*mutS*, *mutL*, *mutY*, *ung*, and *mfd*)

Table 2 Primers used in this study

Primer	Sequence (5′–3′)	Underlined
sphU	GTTGATCGTGCTACTCGGATGCTCAAAGACTCGG	*Sph*I site abolished
sphD	CCGAGTCTTTGAGCATCCGAGTAGCACGATCAAC	*Sph*I site abolished
catF1	GCCGCATGCAATTTAATAAAATTGATTTAG	*Sph*I site
catF2	GCCCATATGCAATTTAATAAAATTGATTTAG	*Nde*I site
catR	GCCGGATCCTTATAAAAGCCAGTCATTAG	*Bam*HI site
138sF	CTATACCTGAAAATTCTTTTTCTCTTTCTATTATTC	Mutation codon
138sR	GAATAATAGAAAGAGAAAAAGAATTTTCAGGTATAG	Mutation codon
138vF	CTATACCTGAAAATGTATTTTCTCTTTCTATTATTC	Mutation codon
138vR	GAATAATAGAAAGAGAAAATACATTTTCAGGTATAG	Mutation codon
T7pro	TAATACGACTCACTATAGGG	
T7term	GCTAGTTATTGCTCAGCGG	

G. kaustophilus, was used as an *E. coli–Geobacillus* shuttle plasmid to induce gene expression in *G. kaustophilus* under the control of the P_{gk704} promoter. The *cat* gene, which encodes *S. aureus* CAT, was amplified from the plasmid pNW33N (Rhee et al. 2007) using primers catF1 and catR. This gene was then cloned between the *Sph*I and *Bam*HI sites of pGKE70 and pGKE75 to yield pGKE70-*cat* and pGKE75-*cat*, respectively. The *cat* gene was also amplified using primers catF2 and catR and cloned between the *Nde*I and *Bam*HI sites of pET-16b (Novagen, Darmstadt, Germany) to yield pET-*cat*.

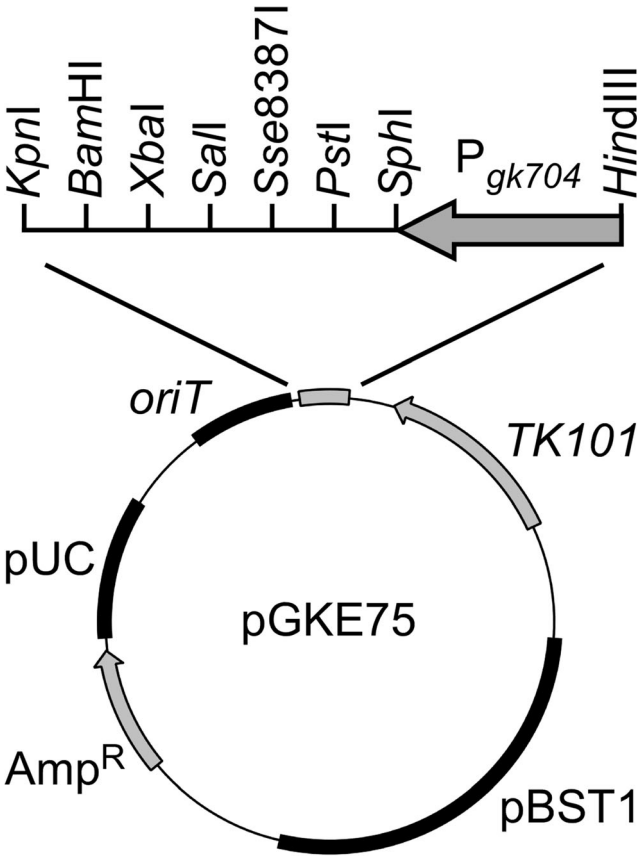

Fig. 1 pGKE75 structure. Plasmid pGKE75, which replicates autonomously, can be shuttled between *E. coli* and *G. kaustophilus*. Amp^R ampicillin resistance gene, pUC pUC replication region, *oriT* conjugative transfer origin, *TK101*, thermostable kanamycin nucleotidyltransferase gene, P_{gk704} the P_{gk704} promoter functional in *G. kaustophilus* (Suzuki et al. 2013b), pBST1 replicon for *G. kaustophilus* (Taylor et al. 2008). Unique restriction enzyme sites within multiple cloning sites are also indicated

Plasmid introduction into *G. kaustophilus*

Derivatives of pGKE70 and pGKE75 were introduced into *G. kaustophilus* cells using conjugative DNA transfer from *E. coli* DH5α (Suzuki and Yoshida 2012; Suzuki et al. 2013a). Transformants carrying pGKE70 derivatives integrated at the *trpE* locus were selected using kanamycin resistance and tryptophan auxotrophy. Transformants carrying pGKE75 derivatives were selected using kanamycin resistance.

Generation of cat^{A138T} mutant in *G. kaustophilus* MK480

G. kaustophilus MK480 harboring pGKE75-*cat* was incubated twice at 65 °C for 24 h on LK5 plates containing 10 mg l^{-1} Cm. The resulting cells (approximately 10^4 cells) were collected and then incubated at 65 °C on LK5 plates containing 20 mg l^{-1} Cm. Similar successive cultures were conducted on LK5 plates containing 30, 40, and 50 mg l^{-1} Cm (Table 3). Plasmid mixtures were isolated from cells resistant to 50 mg l^{-1} Cm, inserted into *E. coli* DH5α, and then reintroduced into *G. kaustophilus* MK242 using conjugative transfer. The transformants were incubated at 60 °C for 24 h on LK5 plates containing 10 mg l^{-1} Cm. At this point, pGKE75-*cat* derivatives were isolated from clones that exhibited obvious Cm resistance. When these plasmids were analyzed for P_{gk704}-*cat* sequences, the plasmid pGKE75-cat^{A138T}, which carries a *cat* mutant (cat^{A138T}), was identified.

Table 3 Cm resistance transition of *G. kaustophilus* MK480 harboring the indicated plasmid

Culture round	Highest Cm concentration that allowed cell growth (mg l^{-1})		Colony formation	
	pGKE75-*cat*	pGKE75	pGKE75-*cat*	pGKE75
1	10	2	Huddled	Huddled
2	10	2	Huddled	Huddled
3	20	3	Huddled	Huddled
4	30	3	Huddled	Huddled
5	40	3	Huddled	Huddled
6	50	5	Substantial	Huddled

Successive culture was repeated at 65 °C for 24 h on LK5 plates with Cm. Cells that exhibited Cm resistance at the indicated concentration were collected, and an aliquot (10^4 cells) was used for the next culture. In rounds 1–5, cells harboring pGKE75-*cat* slightly grew only when spread on plates at high cell density, and the colonies were huddled (Huddled). At round 6, a portion of the cells (1 % of total) formed substantial colonies on LK5 plates containing 50 mg l^{-1} Cm, even when spread at low cell density (substantial)

Generation of cat^{A138S} and cat^{A138V} mutants by in vitro site-directed mutagenesis

The upstream and downstream regions of the *cat* gene were amplified from the plasmid pET-*cat* using primers t7pro and 138sR (for upstream) and 138sF and t7term (for downstream), respectively. The two fragments were fused by overlap extension polymerase chain reaction (PCR) to yield the mutant gene cat^{A138S}. The upstream and downstream regions were also amplified using primers T7pro and 138vR and 138vF and t7term, respectively, and fused to yield the cat^{A138V} gene.

Preparation of recombinant proteins

E. coli BL21-CodonPlus(DE3)-RIPL harboring pET-*cat* was aerobically cultured at 20 °C for 16 h in liquid LB medium (100 ml) supplemented with lactose (1 %). Harvested cells were suspended in lysis buffer (10 mM Tris–HCl, pH 8.0; 10 ml) and sonicated. The cell lysate was clarified by centrifugation to eliminate cell debris and then applied to a TALON metal affinity resin column (Takara Bio, Shiga, Japan; 0.1-ml bed) equilibrated with the same buffer. The column was successively washed with buffers containing 20 mM (1 ml), 50 mM (0.5 ml), 100 mM (0.5 ml), and 150 mM (0.5 ml) imidazole. Recombinant proteins were subsequently eluted with a buffer containing 200 mM imidazole and dialyzed against storage buffer (10 mM Tris–HCl, pH 7.8, 0.2 M NaCl). The mutant *cat* genes cat^{A138T}, cat^{A138S}, and cat^{A138V} were also cloned in pET-16b and used to prepare CAT^{A138T}, CAT^{A138S}, and CAT^{A138V} proteins, respectively, using the same procedure described above for the preparation of CAT. We assessed protein purity using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using the Bradford method with bovine serum albumin as the standard.

CAT activity assay

CAT catalyzes the transfer of an acetyl group from acetyl-CoA to Cm, producing acetylated Cm and CoASH. Standard reaction mixtures (0.3 ml) contained 50 mM sodium phosphate (pH 7.0), 0.1 mM Cm, 0.1 mM acetyl-CoA, and 1 mM 5,5′-dithio-bis(2-nitrobenzoic acid). During the reaction, the product CoASH stoichiometrically reduces 5,5′-dithio-bis(2-nitrobenzoic acid) to produce 5-thio-2-nitrobenzoic acid. The mixture was preincubated for 1 min in a quartz cell inserted into a spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan) equipped with a thermostat (TCC-240A, Shimadzu). Adding an enzyme and monitoring, the absorbance of the solution at 412 nm, which arises from 5-thio-2-nitrobenzoic acid, subsequently initiated the reaction. The concentration of 5-thio-2-nitrobenzoic acid was determined using its molar extinction coefficient at 412 nm (13, 600 M^{-1} cm^{-1}). The value was then used to determine the amount of CoASH produced during the reaction. One unit was defined as the amount of enzyme that produces 1 μmol of CoASH per minute. The specific activity was determined from three independent experiments and is presented as the mean±standard deviation. Kinetic parameters were determined using Lineweaver–Burk analysis from the initial reaction rates when 50–200 μM Cm and 100 μM acetyl-CoA were used as substrates.

Thermostability assay

Protein solutions (50 μl; 0.2 g l^{-1} protein) were incubated at 65–80 °C for 1 h and then centrifuged (16,000×*g* for 10 min at 4 °C) to remove aggregated proteins. CAT activity retained in the supernatant was then analyzed. The temperature at which the residual activity equaled 50 % ($T_{1/2}$) was determined by linear regression of the thermal inactivation curves (CAT, 74–77 °C; CAT^{A138T}, 76–80 °C; CAT^{A138S}, 73–78 °C; CAT^{A138V},

75–79 °C). For time-course analyses, protein solutions were incubated at 75 °C for 6 h, centrifuged, and then the supernatant was assayed for CAT activity.

Gel filtration column chromatography

Gel filtration was performed using an ÄKTA pure 25 system (GE Healthcare Japan) equipped with a Superdex 75 10/300 GL column (GE Healthcare Japan, Tokyo, Japan). Proteins were separated by isocratic elution in buffer (50 mM Tris-HCl, pH 7.0, 0.15 M NaCl) at a flow rate of 0.5 ml min^{-1}.

Homology modeling of CAT structures

We built three-dimensional models of CAT and its variants, with the MODELLR program (http://salilab.org/modeller) using the crystal structure of *E. coli* CAT (PDB ID, 3CLA; sequence identity to *S. aureus* CAT, 41 %) as a template. Intramolecular interactions in the model structures were analyzed using the PIC program (http://pic.mbu.iisc.ernet.in).

Cm resistance assay

G. kaustophilus MK242, harboring either pGKE75-*cat* or pGKE75-cat^{A138T}, was incubated at 50–70 °C for 24 h on LK5 plates with or without Cm (5 mg l^{-1}). The resulting colonies were counted to determine the Cm resistance efficiency, which was defined as the ratio of the number of Cm-resistant colonies (grown on plates with Cm) to the total number of colonies (grown on plates without Cm).

Results

Thermoadaptation-directed evolution using previous procedures

Following the procedure described in the previous report (Suzuki et al. 2015), the *cat* gene was first integrated into the *trpE* locus of *G. kaustophilus* MK480 using plasmid pGKE70-*cat*. The resultant cells grew at 45 °C in liquid LK5 containing 10 mg l^{-1} Cm after 2 days of incubation. However, they did not grow at 60 °C, probably because the CAT protein produced in these cells was inactivated by thermal denaturation. However, these cells became Cm resistant after successive culture at 60 °C in the presence of 1, 5, 15, and 20 mg l^{-1} Cm. The resultant cells formed about 50 substantial colonies on LK5 plates containing 10 mg l^{-1} Cm even at 65 °C. The P_{gk704}-*cat* genes integrated at the *trpE* loci of 18 of these clones were amplified and sequenced. The results showed that none of the sequences contained mutations. This indicates that MK480 cells can become resistant to Cm without *cat* mutations through successive culture in the presence of the antibiotic.

Thermoadaptation-directed evolution using pGKE75-*cat*

To promptly distinguish between thermoadaptation-directed *cat* mutations and other factors responsible for Cm resistance at high temperatures, we exploited pGKE75-*cat*. This plasmid is capable of autonomous replication in both *E. coli* and *G. kaustophilus* and thereby shuttles between these bacteria. *G. kaustophilus* MK480 harboring pGKE75-*cat* was successively cultured at 65 °C in liquid LK5 containing 10–100 mg l^{-1} Cm. Successive culture was repeated over 13 times, generating cells stably resistant to 15 mg l^{-1} Cm at 70 °C. The pGKE75-*cat* derivatives were extracted from this cell mixture and reintroduced in *G. kaustophilus* MK242, which is genetically stable, unlike MK480. However, when the transformants were incubated at 60 °C on LK5 plates containing 10 mg l^{-1} Cm, no cells exhibiting obvious Cm resistance were identified. We also analyzed the P_{gk704}-*cat* sequences from several pGKE75-*cat* derivatives and confirmed that none of the plasmids contained mutations. These results suggest that pGKE75-based mutations can facilitate the elimination of false-positive clones (Cm-resistant cells because of factors other than *cat* mutations) and that successive culture in liquid media may be unsuitable for generating *cat* mutations.

Thermoadaptation-directed evolution using pGKE75-*cat* and solid media

Cells producing functional CAT proteins inactivate Cm by acetylation and thereby reduce the concentration of intact Cm in the immediate environment. In liquid medium, however, Cm diffuses and contacts all cells equally. This may result in a weak growth bias for cells producing functional CAT. Based on this hypothesis, we cultured *G. kaustophilus* MK480 harboring pGKE75-*cat* on LK5 plates supplemented with Cm (Table 3). Until the fifth culture, cells grew poorly, with huddled colonies, only when spread on the plates at high cell density. However, at the sixth culture, a few of the cells (about 1 % of the total) formed substantial colonies when spread at low density. At that point, the pGKE75-*cat* derivatives were extracted from the cells and reintroduced into *G. kaustophilus* MK242 to eliminate false-positive clones. The cells were incubated at 60 °C on LK5 plates containing 10 mg l^{-1} Cm to check for Cm resistance. A total of 16 clones obviously resistant to Cm were identified. The pGKE75-*cat* derivatives were isolated from eight clones of these clones, and their P_{gk704}-*cat* sequences were analyzed. All eight carried the cat^{A138T} mutant gene, which contains one mutation (G412A) responsible for an A138T replacement in the amino

acid sequence (Fig. 2a). These plasmids had no mutations in the P_{gk704} promoter region.

Thermostability of the CAT and CAT^{A138T} proteins

The *cat* and *cat*^{A138T} genes were expressed in *E. coli* cells. The gene products, which contained *N*-terminal His_{10}-tags, were purified to homogeneity by immobilized metal ion affinity chromatography (Fig. 2b). To assess their thermostability, protein solutions were incubated at elevated temperatures and analyzed for residual CAT activity (Fig. 3a). The CAT protein was completely inactivated when incubated at 77 °C, whereas CAT^{A138T} activity was substantially retained (47 %). Complete inactivation of CAT^{A138T} was observed at 80 °C. The $T_{1/2}$ values of CAT and CAT^{A138T} were 74.5 and 76.9 °C, respectively. In a time-course analysis, CAT was completely inactivated after 3 h of incubation at 75 °C, whereas CAT^{A138T} retained 45 % of its activity (Fig. 3b). These results show that CAT^{A138T} is more thermostable than CAT.

Catalytic activity of the CAT and CAT^{A138T} proteins

The catalytic activities of CAT and CAT^{A138T} were analyzed at 30–70 °C. The enzymes exhibited almost identical temperature profiles, with their highest activity at 50–60 °C (Fig. 3c). The specific activities of CAT and CAT^{A138T} at 60 were 210±5 and 200±9 U mg^{-1}, respectively. The kinetic parameters of CAT (k_{cat}, 209±17 s^{-1}; K_m for Cm, 162±17 μM) and CAT^{A138T} (k_{cat}, 193±41 s^{-1}; K_m for Cm, 153±50 μM) were also comparable at 60 °C. Thus, the A138T substitution had no effect on CAT activity.

Cm resistance conferred by the *cat* and *cat*^{A138T} genes

G. kaustophilus MK242 carrying pGKE75-*cat* or pGKE75-*cat*^{A138T} was analyzed for Cm resistance efficiency at 50–70 °C (Fig. 4). The efficiency conferred by *cat*^{A138T} was twice that conferred by *cat* at 55–60 °C, although the *cat* gene conferred a slightly higher efficiency than *cat*^{A138T} at 50 °C. Neither *cat* nor *cat*^{A138T} conferred substantial Cm resistance above 65 °C. These data indicated that CAT^{A138T} is also more thermostable than CAT in vivo.

Enzymatic properties of CAT^{A138S} and CAT^{A138V} variants

To determine how the A138T replacement contributes to CAT^{A138T} thermostability, two CAT variants (CAT^{A138S} and CAT^{A138V}; Fig. 2) were prepared and their thermostability and activities were assessed. As shown in Fig. 5, CAT^{A138S} and CAT^{A138V} were more thermostable than CAT, with $T_{1/2}$ values of 75.3 and 76.5 °C, respectively. This result suggests that both of the hydroxyl group and the bulk of the threonine side chain contribute to CAT^{A138T} thermostability. With regard to specific activity, CAT^{A138V} had a somewhat higher specific activity at 60 °C (240±7 U mg^{-1}) than CAT and CAT^{A138T}, whereas CAT^{A138S} had a somewhat lower specific activity (140±6 U mg^{-1}).

Subunit structures of CAT and its variants

Gel filtration analysis of recombinant CAT, CAT^{A138T}, CAT^{A138S}, and CAT^{A138V} proteins showed that all four exhibited single protein peaks with retention volumes almost identical to that of the standard conalbumin (75 kDa). This result is consistent with previous observations (Shaw and Brodsky 1968). Therefore, it is likely that these proteins form homotrimers, as does *E. coli* CAT (Leslie et al. 1988; Leslie 1990).

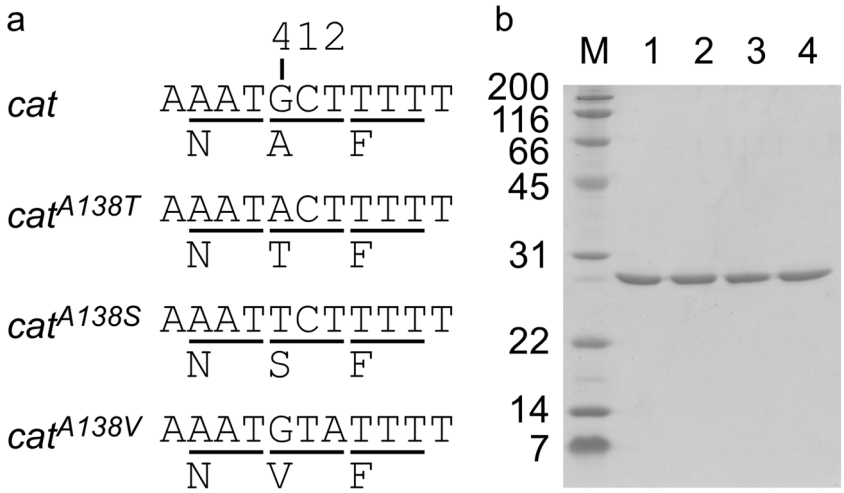

Fig. 2 Preparation of CAT protein and its variants. **a** Mutations in *cat*^{A138T}, *cat*^{A138S}, and *cat*^{A138V} genes are shown in comparison with the *cat* gene. Deduced amino acid sequences are also indicated with underlined codons. **b** Purified recombinant CAT (*lane 1*), CAT^{A138T} (*lane 2*), CAT^{A138S} (*lane 3*), and CAT^{A138V} (*lane 4*) proteins were analyzed by SDS-PAGE with molecular markers (*lane M*; kDa)

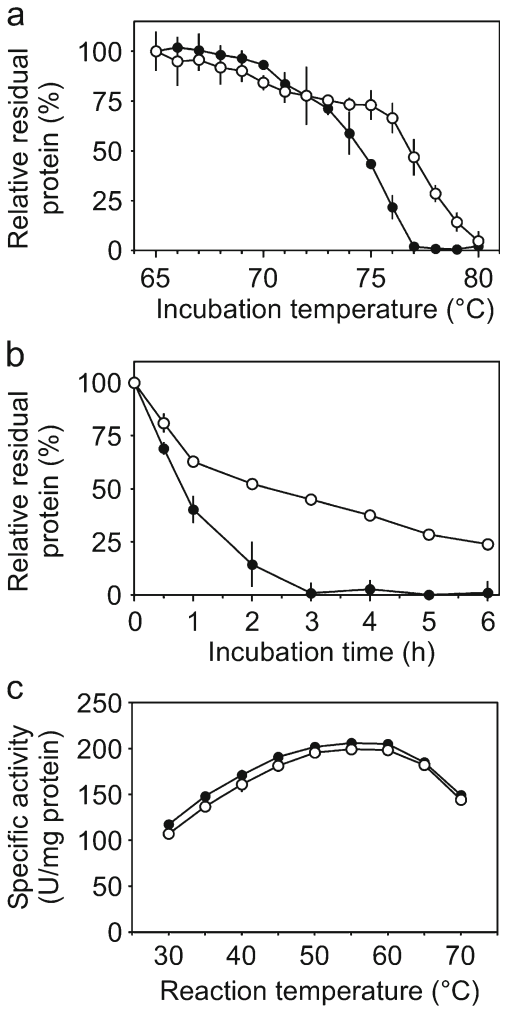

Fig. 3 Thermal inactivation curves (**a**), time-course of inactivation (**b**), and temperature-dependent specific activity (**c**) of CAT (*solid circles*) and CAT^{A138T} (*hollow circles*). **a** After the protein solution was incubated at the indicated temperatures for 1 h, CAT activity retained in the supernatant was analyzed. The activity remaining after incubation at 65 °C was considered to be 100 %. **b** After the protein solution was the incubated at 75 °C for the indicated amount of time, CAT activity retained in the supernatant was analyzed. The activity without incubation was considered to be 100 %. **c** CAT-specific activity was determined at the indicated temperatures. Data are presented as the mean±standard deviation (n=3)

Discussion

Based on the known mutation frequency in *G. kaustophilus* MK480 (Suzuki et al. 2015), this strain presumably generates 10^{-5}–10^{-4} mutations per gene, per culture. Thus, culture of this strain produces minor cell populations (<0.01 %) that may have a mutation in a gene and a major cell population (>99.99 %) that does not. This mutation frequency seems low, but it could allow the generation of a substantial number of mutants (e.g., 10^2–10^3 mutants from 10^7 cells). In this study, we examined the thermoadaptation-directed evolution of *S. aureus* CAT in the MK480 strain and successfully

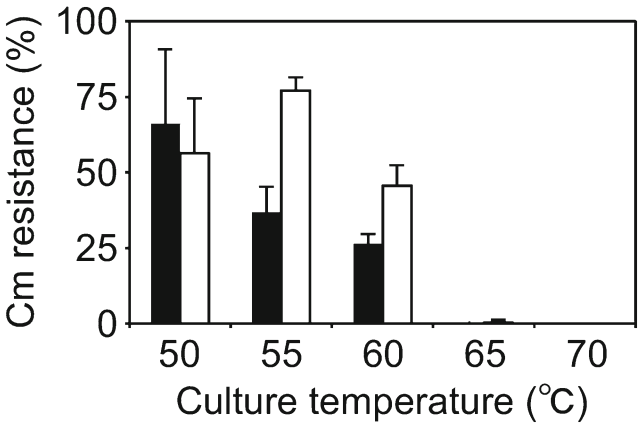

Fig. 4 Cm resistance conferred by the *cat* and cat^{A138T} genes. *G. kaustophilus* MK242 cells harboring pGKE75-*cat* (*solid bars*) or pGKE75-cat^{A138T} (*hollow bars*) were incubated at 50–70 °C on LK5 plates with or without Cm (5 mg l^{-1}). The Cm resistance efficiency was defined as the ratio of the number of Cm-resistant cells to the total number of cells. Data are presented as the mean±standard deviation (n=4). *G. kaustophilus* MK242 cells harboring pGKE75 had <0.1 % efficiency at all temperatures examined

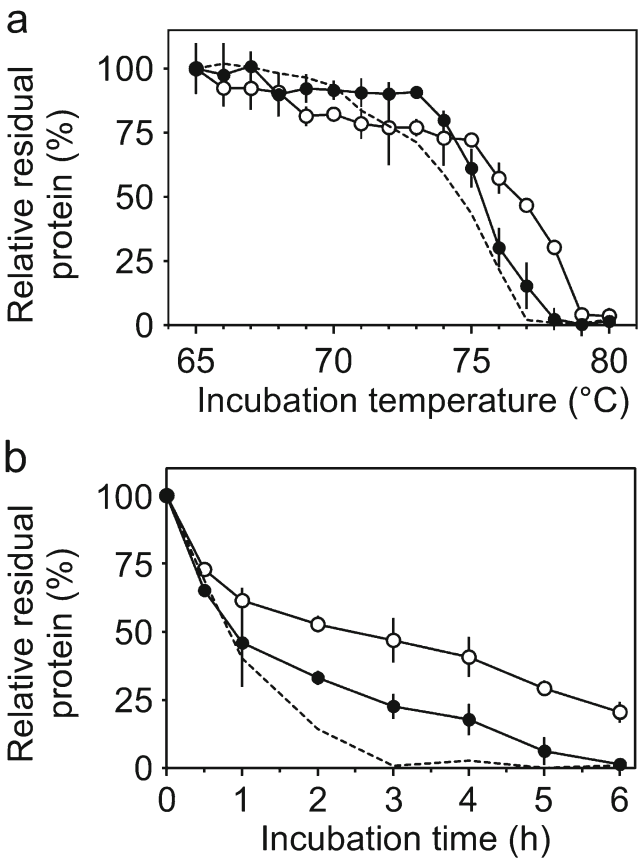

Fig. 5 Thermal inactivation curves (**a**) and time-course of inactivation (**b**) of CAT^{A138S} (*solid circles*), CAT^{A138V} (*hollow circles*), and CAT (*dashed lines*). **a** After the protein solution was incubated at the indicated temperatures for 1 h, CAT activity retained in the supernatant was analyzed. The activity remaining after incubation at 65 °C was considered to be 100 %. **b** After the protein solution was incubated at 75 °C for indicated time, CAT activity retained in the supernatant was analyzed. The activity without incubation was considered to be 100 %

generated the mutant gene cat^{A138T} by six successive cultures using improved procedures (i.e., plasmid-based mutations on solid media). The MK480 cells formed substantial colonies beginning with the sixth culture. This observation indicates that the cat^{A138T} mutant was generated during the fifth or sixth culture by just a matter of odds and that no mutants encoding thermostable CAT variants were generated before then. As expected, enzymatic characterization showed that the CAT^{A138T} protein is more thermostable than CAT. Moreover, the cat^{A138T} gene conferred Cm resistance to *G. kaustophilus* MK242 more efficiently than did the *cat* gene (Fig. 4). The *cat* gene has been used as a selectable marker for *Geobacillus* spp., but successful selection requires either an inconvenient two-step incubation (e.g., at 50 °C followed by 60 °C) or a long-term incubation (e.g., at 48 °C for 5 days) (Imanaka et al. 1982; Wu and Welker 1989). The cat^{A138T} gene may, therefore, be more useful than *cat* as a selectable marker for genetic modifications in *Geobacillus* spp. Notably, CAT^{A138T} maintained efficient catalytic activity comparable to that of CAT (Fig. 3). This result shows that thermoadaptation-directed evolution using the MK480 strain can generate a mutation that enhances enzyme thermostability without affecting catalytic activity.

CAT members are currently classified into three types on the basis of their substrate specificities (Biswas et al. 2012). *S. aureus* CAT is classified as type III. The three-dimensional structure of another type III CAT, from *E. coli*, has been determined by X-ray crystallographic analysis (Leslie et al. 1988; Leslie 1990). We used homology modeling based on a crystal structure of *E. coli* CAT to create structural models of *S. aureus* CAT and CAT^{A138T} and then used these models to analyze the molecular mechanisms responsible for the enhancement of thermostability by the A138T replacement. In the model of *S. aureus* CAT (Fig. 6a), residue A138 resides on β-sheet 7 and is located on the protein surfaces far from the active center. Although the side chains of I134, P135, and L164 surround this residue, no hydrophobic interactions were observed within 4 Å. However, in the model of CAT^{A138T} (Fig. 6b), the hydroxyl group of T138 forms hydrogen-bonding interactions with the backbone amides of residues E84 (oxygen atom) and L86 (nitrogen atom). In addition, the methyl group in the T138 side chain appears to gather I134, P135, and L164 side chains using hydrophobic interactions. Because CAT^{A138S} and CAT^{A138V} showed higher thermostability than CAT, we also constructed models of CAT^{A138S} and CAT^{A138V}. In the initial CAT^{A138S} model (Fig. 6c), the hydroxyl group of S138 formed no hydrogen bonds. However, hydrogen bonds were possible with the backbone amides of residue E84 when the hydroxyl group was rotated. In the CAT^{A138V} model (Fig. 6d), hydrophobic interactions were observed within 4 Å among the side chains of V138, I134, P135, and L164. Taken together, these models suggest that the mutations in CAT^{A138S} and CAT^{A138V} enhanced thermostability by improving hydrogen-bonding and hydrophobic interactions, respectively. Likewise, the mutation in CAT^{A138T} enhanced thermostability by improving both interactions, thereby making the structure more robust. Meanwhile, it is likely that the A138T replacement had no effect on the flexibility of the active center, because CAT^{A138T} maintained efficient activity.

We note that the in vitro random mutagenesis approach could potentially identify the A138T replacement as a thermostability enhancement for the CAT protein. However, spontaneous mutation in the MK480 strain has the following advantages for generating thermostable enzyme variants, compared with in vitro random mutagenesis. During directed evolution, mutant libraries are constructed with low mutation frequency (a few mutations per gene), which is preferred because enzymes retaining function appear to decline exponentially with increasing numbers of amino acid substitutions (Bloom et al. 2005; Drummond et al. 2005). Whereas only a small number of random mutations can be made at a time using in vitro random mutagenesis (Bloom et al. 2005), MK480 cells generate mutations only through cultivation, without the construction of random mutant libraries. This allows simple operations and the generation of a substantial number of mutants while maintaining a low mutation frequency. Moreover, MK480 cells can simultaneously select active thermostable clones using appropriate selection pressures and high-temperature culture. Thus, thermoadaptation-directed evolution using the

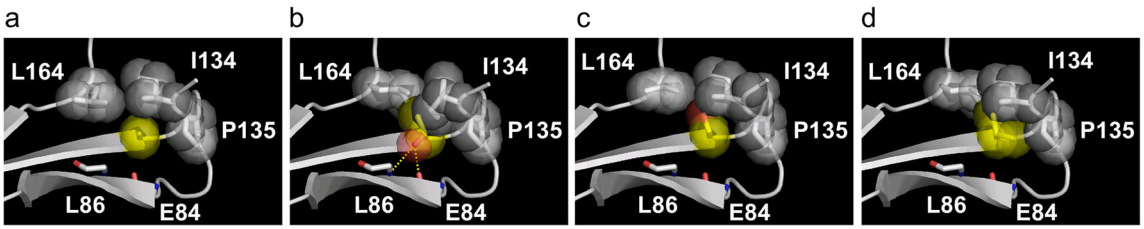

Fig. 6 Structural models of the area surrounding residue 138 of CAT (**a**), CAT^{A138T} (**b**), CAT^{A138S} (**c**), and CAT^{A138V} (**d**). The structures were constructed using homology modeling and rendered using PyMOL software. The side chains of I134, P135, L164, and residue 138 are shown using sticks with spheres. Residue 138 is shown in *yellow*. Sticks show the backbone amide bonds of L86 and E84. Oxygen and nitrogen atoms are rendered in *red* and *blue*, respectively. Hydrogen bonds involving the hydroxyl group of T138 are shown as *dashed lines* (color figure online)

MK480 strain is convenient for readily identifying a crucial mutation for moderate thermostability enhancement.

Spontaneous mutagenesis rarely causes adjacent mutations within one amino acid codon and hence seldom generates several amino acid replacements (e.g., only a GCT → TGG mutation responsible for Ala → Trp replacement). However, once a crucial mutation is identified, further enhancement of thermostability and improvement in catalytic activity may be possible via the saturation mutagenesis approach targeting the original mutation site (Asako et al. 2008; Labrou 2010). This idea is supported by the fact that the CAT^{A138V} variant exhibited somewhat higher activity than CAT or CAT^{A138T}. In vitro random mutagenesis, which can generate mutations with high frequency and thereby explore multiple interacting mutations inaccessible to low frequency mutagenesis (Drummond et al. 2005), is also a powerful approach for further enhancement of thermostability. Moreover, when the *S. aureus* CAT structure is elucidated, it may be possible to enhance CAT^{A138T} thermostability via a rational design approach, optimizing the amino acid configuration surrounding the T138 residue. Therefore, cooperation among protein engineering, using the MK480 strain and these other approaches, would further expand the opportunities for generating useful thermostable enzymes.

In a previous study (Suzuki et al. 2015), mutations occurred at a gene in the MK480 chromosome. However, *cat* mutations were generated only when cultured on solid media. Although it is unclear why growth on solid media was efficient for mutations, definite growth biases based on selective pressures could be an important advantage of solid media. In addition to the strategic switch from liquid to solid media, the identification of cat^{A138T} required a switch from chromosome-based mutations to plasmid-based mutations. Because plasmids can be readily isolated and reintroduced into fresh host cells to examine the activity and thermostability of encoded proteins, in contrast to genes integrated into chromosomes, this strategy efficiently eliminated false-positive clones that adapted to Cm by factors other than *cat* mutation. Moreover, plasmids enabled direct sequencing of target genes without preliminary PCR amplification. This avoids PCR errors, which are indistinguishable from gene mutations during successive culture. The fact that the generation of cat^{A138T} was only achieved by using the improved procedure suggests its crucial role in certain cases.

This study has also revealed two new challenges for thermoadaptation-directed evolution using the MK480 strain. One challenge is the formation of biased mutations. Together with the previous study, which generated orotidine-5′-phosphate decarboxylase variants (Suzuki et al. 2015), two C/G → T/A transitions and one C/G → A/T transversion occurred in the target genes. Such biased mutations reflect intrinsically frequent C/G → T/A transition in *G. kaustophilus* (Suzuki et al. 2015). For a more equal distribution of mutations, it may be effective to expose cells to mutagenic agents such as hydrogen peroxide or *N*-ethyl-*N*-nitrosourea during successive culture. Mutagenic agents could also enhance mutation frequencies.

The other challenge is involuntary adaptation. Although MK480 cells harboring pGKE75-cat^{A138T} were able to grow in media containing 50 mg l^{-1} Cm at 65 °C, MK242 cells that were freshly transformed with pGKE75-cat^{A138T} could not grow under the conditions. In also thermoadaptation-directed evolution using previous procedures or pGKE75-*cat* with liquid media, MK480 cells harboring *cat* gene acquired apparent Cm resistance without *cat* mutations. These observations suggest that *G. kaustophilus* can adapt to Cm due to factors other than *cat* mutations. The plasmid-based method effectively eliminated false-positive clones that adapted to Cm, but such adaptation is still troublesome because it reduces selective pressure. This adaptation may involve rRNA gene mutations that circumvent Cm actions targeting the bacterial 23S ribosome (Mankin and Garrett 1991) or the activation of drug efflux pumps responsible for extracellular elimination of Cm (McMurry et al. 1994). However, it more likely arises from the induction of protein chaperones (or chaperone-like factors) that suppress CAT thermal denaturation because MK480 cells without the *cat* gene remained sensitive to Cm even after similar successive culture (Table 3). The identification of protein chaperones and/or chaperone-like factors induced in MK480 cells may be helpful in clarifying the mechanism of adaptation. This may also help to overcome the adaptation challenge and extend the utility of thermoadaptation-directed enzyme evolution in strain MK480.

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References

Asako H, Shimizu M, Itoh N (2008) Engineering of NADPH-dependent aldo-keto reductase from *Penicillium citrinum* by directed evolution to improve thermostability and enantioselectivity. Appl Microbiol Biotechnol 80(5):805–812. doi:10.1007/s00253-008-1594-z

Ben Mabrouk S, Ayadi DZ, Ben Hlima H, Bejar S (2013) Thermostability improvement of maltogenic amylase MAUS149 by error prone PCR. J Biotechnol 168(4):601–606. doi:10.1016/j.jbiotec.2013.08.026

Biswas T, Houghton JL, Garneau-Tsodikova S, Tsodikov OV (2012) The structural basis for substrate versatility of chloramphenicol acetyltransferase CAT_I. Protein Sci 21(4):520–530. doi:10.1002/pro.2036

Bloom JD, Meyer MM, Meinhold P, Otey CR, MacMillan D, Arnold FH (2005) Evolving strategies for enzyme engineering. Curr Opin Struct Biol 15(4):447–452. doi:10.1016/j.sbi.2005.06.004

Brouns SJJ, Wu H, Akerboom J, Turnbull AP, de Vos WM, van der Oost J (2005) Engineering a selectable marker for hyperthermophiles. J Biol Chem 280(12):11422–11431. doi:10.1074/jbc.M413623200

Drummond DA, Iverson BL, Georgiou G, Arnold FH (2005) Why high-error-rate random mutagenesis libraries are enriched in functional and improved proteins. J Mol Biol 350(4):806–1816. doi:10.1016/j.jmb.2005.05.023

Fang ZM, Zhou P, Chang F, Yin Q, Fang W, Yuan J, Zhang X, Xiao Y (2014) Structure-based rational design to enhance the solubility and thermostability of a bacterial laccase Lac15. PLoS One 9(7): e102423. doi:10.1371/journal.pone.0102423

Fridjonsson O, Watzlawick H, Mattes R (2002) Thermoadaptation of α-galactosidase AgaB1 in *Thermus thermophilus*. J Bacteriol 184(12): 3385–3391. doi:10.1128/JB.184.12.3385-3391.2002

Hoseki J, Yano T, Koyama Y, Kuramitsu S, Kagamiyama H (1999) Directed evolution of thermostable kanamycin-resistance gene: a convenient selection marker for *Thermus thermophilus*. J Biochem 126(5):951–956

Huang J, Cheng Y, Ko T, Lin C, Lai H, Chen C, Ma Y, Zheng Y, Huang C, Zou P, Liu J, Guo R (2012) Rational design to improve thermostability and specific activity of the truncated *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase. Appl Microbiol Biotechnol 94(1):111–121. doi:10.1007/s00253-011-3586-7

Imanaka T, Fujii M, Aramori I, Aiba S (1982) Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. J Bacteriol 149(3):824–830

Labrou NE (2010) Random mutagenesis methods for *in vitro* directed enzyme evolution. Curr Protein Pept Sci 11(1):91–100. doi:10.2174/138920198868622037

Leslie AGW (1990) Refined crystal structure of type III chloramphenicol acetyltransferase at 1.75 Å resolution. J Mol Biol 213(1):167–186. doi:10.1016/s0022-2836(05)80129-9

Leslie AGW, Moody PCE, Shaw WV (1988) Structure of chloramphenicol acetyltransferase at 1.75-Å resolution. Proc Natl Acad Sci U S A 85(12):4133–4137. doi:10.1073/pnas.85.12.4133

Liao H, McKenzie T, Hageman R (1986) Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. Proc Natl Acad Sci U S A 83(3):576–580. doi:10.1073/pnas.83.3.576

Mankin AS, Garrett RA (1991) Chloramphenicol resistance mutations in the single 23S rRNA gene of the archaeon *Halobacterium halobium*. J Bacteriol 173(11):3559–3563

Matsumura M, Aiba S (1985) Screening for thermostable mutant of kanamycin nucleotidyltransferase by the use of a transformation system for a thermophile, *Bacillus stearothermophilus*. J Biol Chem 260(28):15298–15303

McMurry LM, George AM, Levy SB (1994) Active efflux of chloramphenicol in susceptible *Escherichia coli* strains and in multiple-antibiotic-resistant (Mar) mutants. Antimicrob Agents Chemother 38(3):542–546. doi:10.1128/AAC.38.3.542

Nakamura A, Takakura Y, Kobayashi H, Hoshino T (2005) *In vivo* directed evolution for thermostabilization of *Escherichia coli* hygromycin B phosphotransferase and the use of the gene as a selection marker in the host-vector system of *Thermus thermophilus*. J Biosci Bioeng 100(2):158–163. doi:10.1263/jbb.100.158

Rhee MS, Kim J, Qian Y, Ingram LO, Shanmugam KT (2007) Development of plasmid vector and electroporation condition for gene transfer in sporogenic lactic acid bacterium, *Bacillus coagulans*. Plasmid 58(1):13–22. doi:10.1016/j.plasmid.2006.11.006

Shaw WV, Brodsky RF (1968) Characterization of chloramphenicol acetyltransferase from chloramphenicol-resistant *Staphylococcus aureus*. J Bacteriol 95(1):28–36

Stephens DE, Khan FI, Singh P, Bisetty K, Singh S, Permaul K (2014) Creation of thermostable and alkaline stable xylanase by DNA shuffling. J Biotechnol 187:139–146. doi:10.1016/j.jbiotec.2014.07.446

Suzuki H, Yoshida K (2012) Genetic transformation of *Geobacillus kaustophilus* HTA426 by conjugative transfer of host-mimicking plasmids. J Microbiol Biotechnol 22(9):1279–1287. doi:10.4014/jmb.1203.03023

Suzuki H, Murakami A, Yoshida K (2012) Counterselection system for *Geobacillus kaustophilus* HTA426 through disruption of *pyrF* and *pyrR*. Appl Environ Microbiol 78(20):7376–7383. doi:10.1128/aem. 01669-12

Suzuki H, Wada K, Furukawa M, Doi K, Ohshima T (2013a) A ternary conjugation system for the construction of DNA libraries for *Geobacillus kaustophilus* HTA426. Biosci Biotechnol Biochem 77(11):2316–2318. doi:10.1271/bbb.1304921

Suzuki H, Yoshida K, Ohshima T (2013b) Polysaccharide-degrading thermophiles generated by heterologous gene expression in *Geobacillus kaustophilus* HTA426. Appl Environ Microbiol 79(17):5151–5158. doi:10.1128/AEM. 01506-13

Suzuki H, Kobayashi J, Wada K, Furukawa M, Doi K (2015) Thermoadaptation-directed enzyme evolution in an error-prone thermophile derived from *Geobacillus kaustophilus* HTA426. Appl Environ Microbiol 81(1):149–158. doi:10.1128/AEM. 02577-14

Takami H, Takaki Y, Chee G-J, Nishi S, Shimamura S, Suzuki H, Matsui S, Uchiyama I (2004) Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. Nucleic Acids Res 32(21):6292–6303. doi:10.1093/nar/gkh970

Tamakoshi M, Nakano Y, Kakizawa S, Yamagishi A, Oshima T (2001) Selection of stabilized 3-isopropylmalate dehydrogenase of *Saccharomyces cerevisiae* using the host-vector system of an extreme thermophile, *Thermus thermophilus*. Extremophiles 5(1):17–22. doi:10.1007/s007920000168

Taylor MP, Esteban CD, Leak DJ (2008) Development of a versatile shuttle vector for gene expression in *Geobacillus* spp. Plasmid 60(1):45–52. doi:10.1016/j.plasmid.2008.04.001

Wu L, Welker NE (1989) Protoplast transformation of *Bacillus stearothermophilus* NUB36 by plasmid DNA. J Gen Microbiol 135(5):1315–1324. doi:10.1099/00221287-135-5-1315