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A journey down to hell: new thermostable protein-tags for biotechnology at high temperatures

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Abstract

The specific labelling of proteins in recent years has made use of self-labelling proteins, such as the SNAP-tag® and the Halotag®. These enzymes, by their nature or suitably engineered, have the ability to specifically react with their respective substrates, but covalently retaining a part of them in the catalytic site upon reaction. This led to the synthesis of substrates conjugated with, e.g., fluorophores (proposing them as alternatives to fluorescent proteins), but also with others chemical groups, for numerous biotechnological applications. Recently, a mutant of the OGT from *Saccharolobus solfataricus* (H^5) very stable to high temperatures and in the presence of physical and chemical denaturing agents has been proposed as a thermostable SNAP-tag® for in vivo and in vitro harsh reaction conditions. Here, we show two new thermostable OGTs from *Thermotoga neapolitana* and *Pyrococcus furiosus*, which, respectively, display a higher catalytic activity and thermostability respect to H^5, proposing them as alternatives for in vivo studies in these extreme model organisms.

Keywords (Hyper)thermophiles · Thermostable proteins · Protein-tag · Biotechnology

Introduction

Protein-tags are short or long peptide sequences genetically fused to recombinant proteins for various purposes, as affinity purification, protein localization and general labelling procedures. In some cases, the presence of these tags enhances the solubilization of proteins and enzymes expressed in chaperone-deficient species such as *Escherichia coli*, to assist in the proper folding of proteins and avoid precipitation. These include commercially available thioredoxin (TRX), poly(NANP), maltose-binding protein (MBP), and glutathione *S*-transferase (GST). The discovery of Fluorescent Proteins (FPs) has revolutionized the world of cell and molecular biology, allowing several applications as reporter gene in fluorescence microscopy (Chalfie et al. 1994; Tsien 1998; Aliye et al. 2015). Although FPs are intrinsically fluorescent without the addition of any external substrate, they have some disadvantages. Indeed, their relatively large dimensions and the sensitivity to environmental changes (pH or the absence of O_2) affect the formation of the internal natural fluorophore (Ashby et al. 2004; Campbell and Choy 2000).

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Rosanna Mattossovich and Rosa Merlo equally contributed to the present work.

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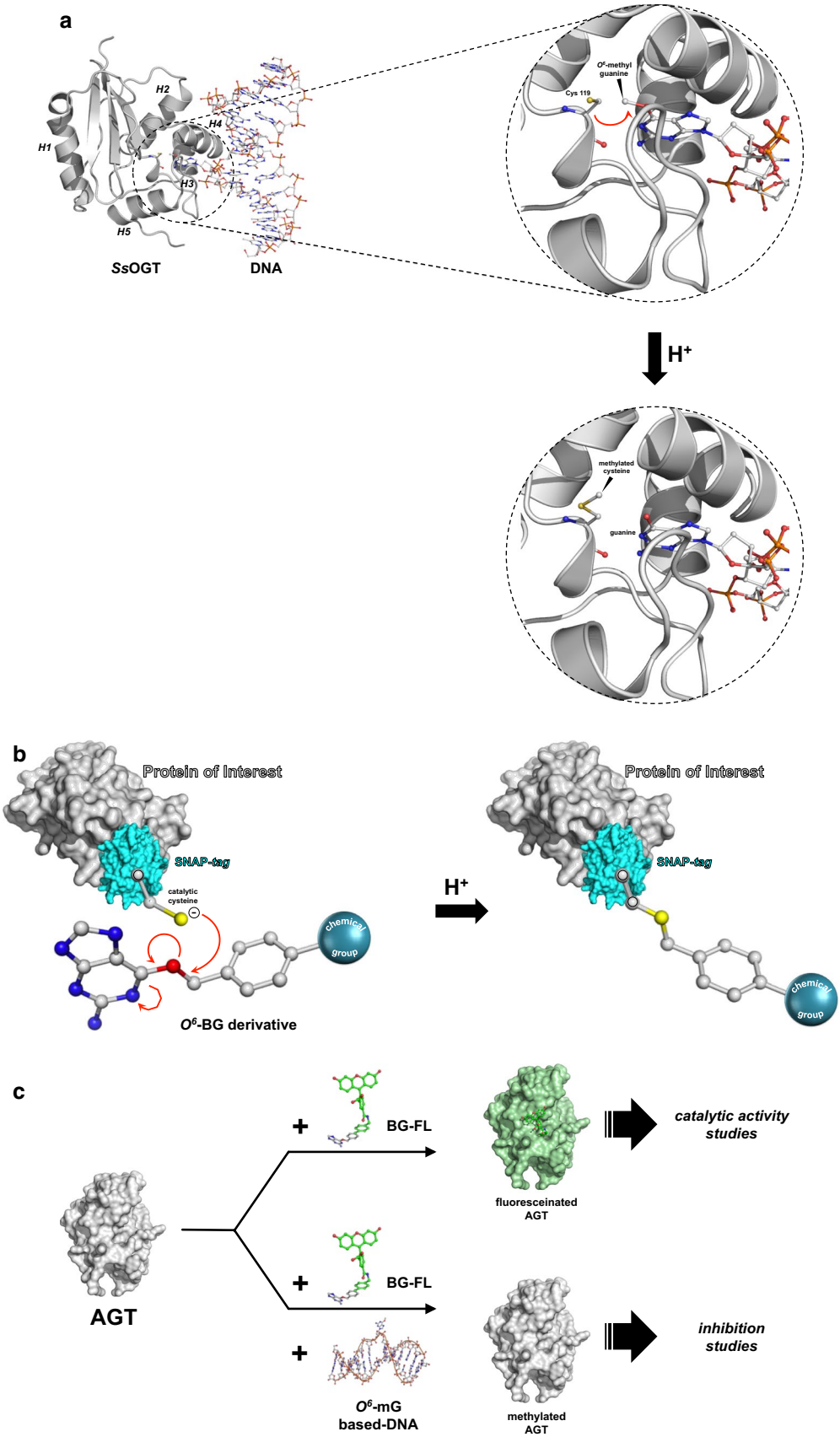

a
H2
H4
H1
H3
H5
SsOGT
DNA
O6-methyl guanine
Cys 119
H+
methylated cysteine
guanine
b
Protein of Interest
SNAP-tag
catalytic cysteine
chemical group
O6-BG derivative
H+
Protein of Interest
SNAP-tag
chemical group
c
AGT
+
BG-FL
+
BG-FL
+
O6-mG based-DNA
fluoresceinated AGT
methylated AGT
catalytic activity studies
inhibition studies

◂**Fig. 1** **a** A cartoon of the AGTs irreversible reaction mechanism is shown as example: the *Ss*OGT enzyme (PDB ID: 4ZYE) recognizes the methylated guanine on DNA (from PDB ID: 4ZYD) and proceeds to the irreversible transfer of the methyl group from the guanine to the catalytic cysteine, inactivating the protein (from PDB ID: 4ZYG) (Perugino et al. 2015). **b** The SNAP-tag® technology is based of an engineered variant of the hAGT, which is able to recognize BG-derivative substrates, and irreversibly transferring in its active site a desired chemical group conjugated to the benzyl moiety. Consequently, this allows the labelling of a protein of interest expressed as fusion protein to the SNAP-tag®. **c** The use of commercially available fluorescent AGT substrates led to the setting up of new safety AGT's assay, for the determination of the catalytic activities, or inhibition studies in the presence of natural substrates (e.g., alkylated DNA) or AGT's inhibitors

Recently, studies on O^6-alkylguanine-DNA-alkyl-transferases (AGTs, OGTs or MGMTs; EC: 2.1.1.63) led to the proposal of alternative protein-tags with new behaviours, useful for several applications in many fields. In nature, these ubiquitous small proteins have a crucial role in the direct repair of DNA by alkylating agents (Mishina et al. 2006; Serpe et al. 2019) and the human homolog (hAGT) is target of chemotherapic drugs for its crucial role in many types of tumours (Sun et al. 2018; Aoki and Natsume 2019). Their catalytic activity is based on the recognition of the damage on DNA (an O^6-alkylguanine or an O^4-alkylthymine) and, by a *one-step* reaction mechanism (via SN_2 type), the alkylic group from the damaged base is irreversibly transferred to a cysteine residue in their own active site (Daniels et al. 2000, 2004; Fang et al. 2005; Tubbs et al. 2007; Pegg 2011; Yang et al. 2009) (Fig. 1a). For these reasons, they are called suicide or kamikaze proteins, with a 1:1 stoichiometry of their reaction with the natural substrate. However, some AGTs resulted very reactive with a strong inhibitor, the O^6-benzyl-guanine (O^6-BG), which was used in combination with chemotherapic drugs (Pegg et al. 2001; Luu et al. 2002; Coulter et al. 2007). This information led professor Kai Johnsson and his group to put their effort for the production of a new protein-tag from a variant of the hAGT, introducing the SNAP-tag® technology in the "biotech" scenario (Keppler et al. 2003, 2004; Gronemeyer et al. 2006; Gautier et al. 2008; Mollwitz et al. 2012): upon the irreversible reaction, hAGT keeps the benzyl moiety of the substrate covalently linked to its catalytic cysteine. This makes possible the labelling of this protein (and a relative protein of interest, if expressed as fusions with it) when any chemical group conjugated to the benzyl moiety of O^6-BG (Keppler et al. 2003, 2004; Gautier et al. 2008) (Fig. 1b). Furthermore, the commercially available fluorescent O^6-BG derivatives (as the SNAP-Vista Green® and the SNAP-Cell® TMR Star; New England Biolabs) allowed the development of new applications in the fluorescent microscopy field, and recently of a new fluorescent assay for all the BG-sensitive AGTs, overcoming the traditional, long and unsafe assays for this class of proteins based on HPLC procedures and radioactive substrates (Hishiguro et al. 2008; Perugino et al. 2012). The presence of methylated-DNA in this assay, led to the determination of the activity of AGTs on their natural substrates, by applying the classical enzyme inhibition approaches (Fig. 1c) (Perugino et al. 2012, 2015; Miggiano et al. 2013, 2017; Vettone et al. 2016; Morrone et al. 2017).

All the above-mentioned protein-tags have the disadvantage to be employed in mild reaction conditions and in mesophilic organisms. This was successfully overcome by the introduction of a thermostable OGT from the hyperthermophilic archaea *Saccharolobus solfataricus* (formerly *Sulfolobus solfataricus*). Starting from the studies of the wild-type enzyme (*Ss*OGT), which not only displays the same behaviours of the hAGT on BG derivatives, but is also characterized by an exceptional stability at extremes of temperature, pH, ionic strength and the presence of denaturing agents (Perugino et al. 2012), we developed a DNA-bindingless variant (called H^5), which resulted in a strong "thermostable SNAP-tag®". H^5 was successfully employed in in vivo heterologous expression in thermophilic organisms as *Thermus thermophilus* HB27 and *Sulfolobus islandicus* E233S1 (Vettone et al. 2016; Visone et al. 2017), in fusion with a thermostable β-glycosidase and a *S. solfataricus* reverse gyrase (Vettone et al. 2016; Visone et al. 2017), as well as under extreme reaction conditions as gene reporter in in vitro transcription/translation systems based on *Sulfolobus* cell lysates (Lo Gullo et al. 2019). Furthermore, H^5 was successfully fused to the N-terminal domain of the ice nucleation protein (INPN) from the Gram-negative bacterium *Pseudomonas syringae*, a transmembrane protein useful for the one-step heterologous expression and the in vivo immobilization of proteins of interest on the outer membrane of *E. coli* (Samuelson et al. 2002). This led to the development of the new Anchoring-and-self-labelling-protein-tag (ASL^{tag}) (Merlo et al. 2019), which simultaneously allows the immobilization (by INPN) and the quantitative determination of the yield of an immobilized protein (by the fluorescent assay using H^5). Surprisingly, the presence of the thermostable H^5 between the INPN and a protein of interest resulted in an enhancement of the stability of the latter, as it was the case for the *Sulfurihydrogenibium yellowstonense* α-carbonic anhydrase (Del Prete et al. 2019).

The growing demand to use recent technologies at very extreme conditions in thermophilic bacteria and archaea, such as the in vivo CRISPR-Cas immune systems, leads us to search for new protein-tags with very high activity and thermostability. In this regard, the present work is focussed on the characterization of two new OGTs, from the hyperthermophilic organisms *Thermotoga neapolitana* and *Pyrococcus furiosus*. The purified proteins were compared to *Ss*OGT, showing respect to it exceptional characteristics in terms of enzymatic activity and thermostability.

These results open promising perspectives in the development of new protein-tags to employ in these extreme model organisms.

Materials and methods

Reagents

Fine chemicals were from Sigma-Aldrich, SNAP-Vista Green® fluorescent substrate (hereinafter BG-FL) was from New England Biolabs (Ipswich, MA). SYPRO Orange 5000× (Invitrogen). Synthetic oligonucleotides listed in Table 1 were from Eurofins (Milan, Italy); *Pfu* DNA polymerase was from NZYTech (Portugal). The Bio-Rad protein assay kit (Bio-Rad Pacific) was used for the determination of the protein concentration, using purified BSA as standard.

DNA constructs

The cloning procedures for the construction of *E. coli* expression plasmids were the same for both the proteins: the ORF CTN1690/PF1878, encoding a putative OGT, was amplified from genomic DNA from *Thermotoga neapolitana* DSMZ 4359^T/*Pyrococcus furiosus* JFW02 strain genomic DNA, using Lig5:Lig3 *Tn*OGT/*Pfu*OGT oligonucleotides pairs (listed in Table 1) and directly cloned into the expression vector pHTP1 (NZYTech, Portugal), following the instructions described in the NZYEasy Cloning and Expression kit I (NZYTech, Portugal) manual. The ligation mixture was entirely used to transform commercial *E. coli* DH5α cells (NZY5α Competent Cells-NZYTech, Portugal) and positive clones were confirmed by PCR. Subsequently, a DNA fragment from the resulted pHTP1-*Tn*OGT/pHTP1-*Pfu*OGT was removed by digestion with Nco I restriction endonuclease, and was replaced by a double-stranded oligonucleotides (NZY-His Fwd2 and NZY-His Rev2; Table 1), whose DNA sequence expresses a shorter His_6-tag (MAHHHHHHTG-), similar to that at the N-terminal of the *Ss*OGT protein (Perugino et al. 2012). Positive clones after transformation of the ligation mixture in *E. coli* KRX competent cells were confirmed by DNA sequencing.

Protein purification

*Tn*OGT and *Pfu*OGT were expressed in the *E. coli* BL21 (DE3) cells grown overnight at 37 °C in Luria–Bertani (LB) selective medium supplemented with 50 mg/L kanamycin and 30 mg/L chloramphenicol, and the protein expression was induced with 1.0 mM isopropyl-thio-β-D-galactopyranoside (IPTG), when an absorbance value of 0.5–0.6 $A_{600\ nm}$ was reached. Harvested cells were resuspended 1:3 (w/v) in Buffer A (50 mM phosphate, 300 mM NaCl; pH 8.0) supplemented with 1% Triton X-100 and stored overnight at − 20 °C. After this first step of lysis, the biomass was treated with lysozyme and DNAse for 60 min in ice, followed by a sonication step. Finally, the lysate was centrifuged for 30 min at 60,000×*g* in and the cell extract recovered. To remove *E. coli* contaminants, all cell extracts were incubated 20 min at 70.0 °C and 20 min at 65 °C, respectively, followed by a centrifugation at 13,000×*g* at 4.0 °C; the supernatant was diluted 1:2 (v/v) in purification Buffer A and applied to a Protino Ni–NTA Column 1.0 mL (Macherey–Nagel) for His_6-tag affinity chromatography. After two washing steps of 10 column volumes of Buffer A and 10 column volumes of Buffer A supplemented with 25.0 mM imidazole, the elution was performed in 20 column volumes of buffer A, by applying a linear gradient of 25.0–250.0 mM imidazole. The fractions containing the protein were collected and analysed by SDS-PAGE. *Tn*OGT was dialysed against PBS 1 × buffer (phosphate buffer 20 mM, NaCl 150 mM; pH 7.3), whereas the fractions of the *Pfu*OGT protein were pooled, concentrated and subjected to a further gel-filtration chromatography, using a Superdex 75 10/300 GL column (GE Healthcare Life Sciences). Finally, both the proteins were concentrated and loaded on 15% SDS-PAGE gel to confirm its purity and stored at − 20.0 °C.

Table 1 List of oligonucleotides used in this study

Name	Sequence	Notes
Lig5-*Pfu*OGT	5′-TCAGCAAGGGCTGAGGCCATGGTATTGGAAGTTAGG-3′	Nco I site underlined
Lig3-*Pfu*OGT	5′-CCTCAGCGGAAGCTGAGGTTAGCTTGTCCATCCTTCC-3′	
Lig5-*Tn*OGT	5′-TCAGCAAGGGCTCAGGCCATGGGAGATCGA-3′	Nco I site underlined
Lig3-*Tn*OGT	5′-CCTCAGCGGAAGCTGAGGTTATCGACTACCTCGC-3′	
NZY-His fwd2	5′-catgGCACACCATCACCATCACCATACGGG-3′	Inserting an His_6-tag (MAHHHHHHTG-; underlined) upstream the *Pfu*OGT and *Tn*OGT sequence
NZY-His rev2	5′-catgCCCGTATGGTGATGGTGATGGTGTGC-3′	
Fwd^{m4}	5′-ggcMgtaggcctagcatgacaatctgcattggtgatcacgg-3′	From Perugino et al. (2015); M = O^6-methyl-guanine
Rev4	5′-ccgtgatcaccaatgcagattgtcatgctaggcctaccgcc-3′	From Perugino et al. (2015)

In vitro alkyl-transferase assay

The fluorescent substrate BG-FL was used for the determination of the catalytic activity of all thermostable enzymes analysed, as previously described (Perugino et al. 2012, 2015; Miggiano et al. 2013; Vettone et al. 2016; Visone et al. 2017; Merlo et al. 2019; Del Prete et al. 2019). Briefly, 5.0 µM of protein (ca. 0.1 mg/mL) was incubated with 10.0 µM of BG-FL in Fluo Buffer 1 × (50.0 mM phosphate, 100.0 mM NaCl, 1.0 mM DTT; pH 6.5) at different temperatures and times, as indicated; each reaction was stopped by adding a Laemmli buffer 1 × (formamide 95%; EDTA 20.0 mM; bromophenol 0.05%), followed by denaturation at 100.0 °C and the direct loading of the sample on SDS-PAGE. The gel was first analysed by fluorescence imaging on a VersaDoc 4000™ system (Bio-Rad) by applying as excitation/emission parameters a blue LED/530 bandpass filter, and then was stained by Coomassie. Assuming the irreversible mechanism with 1:1 BG-FL/OGT ratio, fluorescence intensity data were corrected for the amount of loaded protein, and fitted by applying the second-order rate equation, to determine the relative amount of covalently modified protein in time-course experiments. (Gautier et al. 2008; Miggiano et al. 2013; Perugino et al. 2012, 2015).

Competitive assay and IC_{50} calculation

Competitive assay using the fluorescent substrate in the presence of double strands (ds) oligonucleotides pairs (Fwd^{m4}: Rev4; Table 1), containing a single O^6-methyl-guanine, was performed as described (Perugino et al. 2015) to determine the half maximal inhibitory concentration (IC_{50}), that is the concentration of methylated DNA needed to reduce the fluorescence intensity of the OGT band by 50.0%. Reactions incubated at fixed temperatures with increasing concentrations (0.0–10.0 µM) of ds-Fwd^{m4} and keeping constant the BG-FL concentration (5.0 µM) were performed for 10 min at 50.0 °C. Corrected data of fluorescence intensity were fitted with the IC_{50} equation (Perugino et al. 2015; Morrone et al. 2017).

Protein stability analysis

The stability at several conditions of the thermostable OGTs was analysed by the differential scan fluorimetry method (DSF), adapted by a protocol previously described for the *Ss*OGT and relative mutants (Niesen et al. 2007; Perugino et al. 2015; Vettone et al. 2016; Morrone et al. 2017). Triplicates of each condition containing 25.0 µM of enzyme (ca. 0.5 mg/mL) in PBS 1× buffer and SYPRO Orange dye 1× were subjected to a scan of 70 cycles at temperatures from 25.0 to 94.0 °C for 10 min/°C × cycle, and analysed in a Real-Time Light Cycler™ (Bio-Rad). Relative fluorescence data were then normalized to the maximum fluorescence value within each scan. Obtained plots of fluorescence intensity vs temperature displayed sigmoidal curves (typical of a two-state transition), which allowed the determination of the inflection points (T_m values) by fitting the Boltzmann equation (Niesen et al. 2007; Perugino et al. 2015; Vettone et al. 2016; Morrone et al. 2017).

Data analysis and softwares

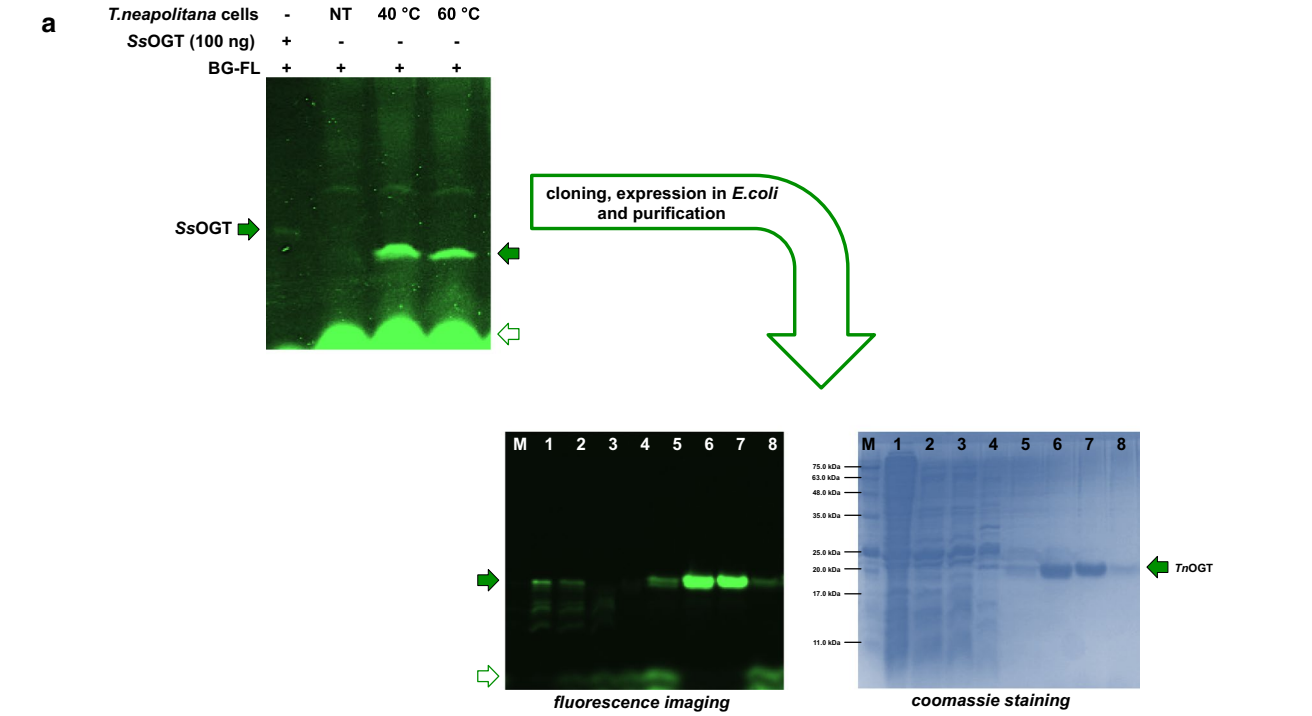
Prism Software Package (GraphPad Software) and GraFit 5.0 Data Analysis Software (Erithacus Software) were used for the data analysis from activity, competitive inhibition and stability assays.

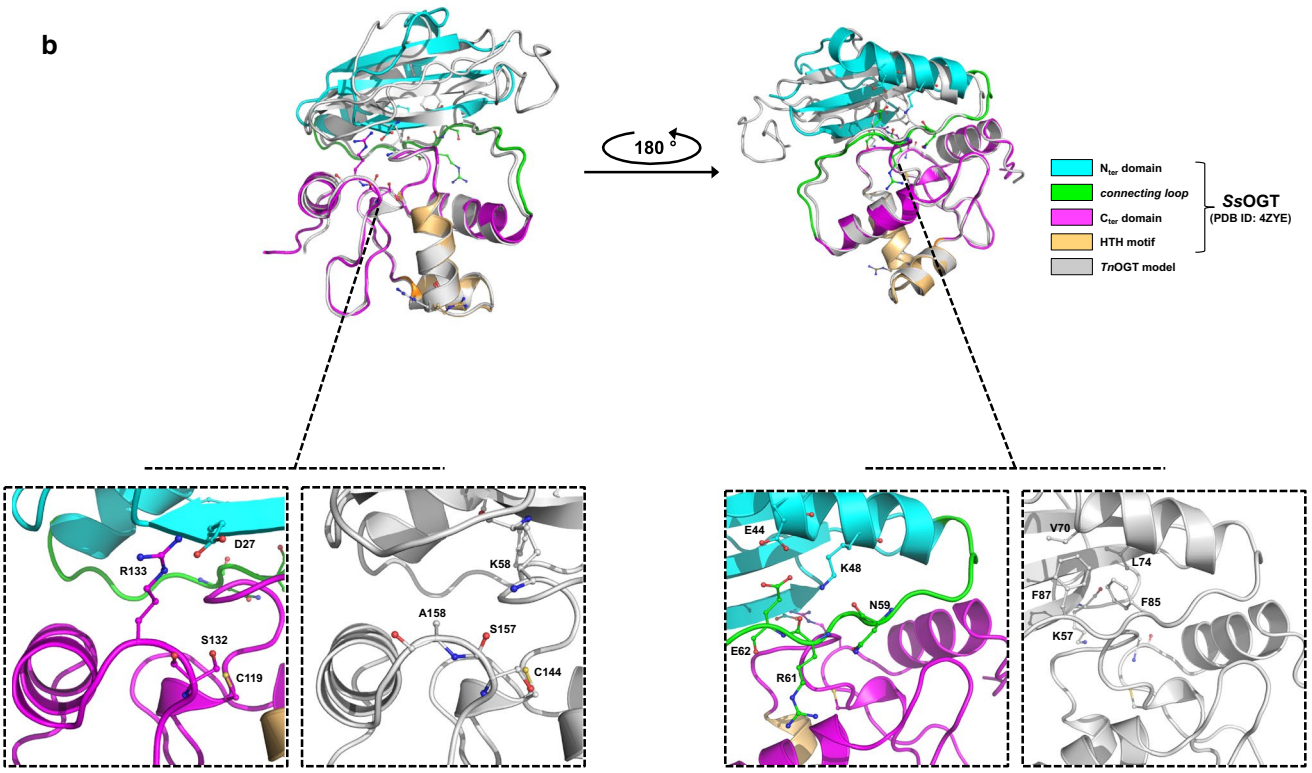
Results and discussion

The OGT from *Thermotoga neapolitana*

Thermotoga neapolitana is a hyperthermophilic Gram-negative bacterium of the order *Thermotogales* (Belkin et al. 1986; Jannasch et al. 1988) that include good candidates for genetic engineering and biotechnological applications (Conners et al. 2006; Zhang et al. 2015; Fink et al. 2016; Donaldson et al. 2017; Han and Xu 2017). Several Thermotogales have been studied for the fermentative production of hydrogen (H_2) by [FeFe] hydrogenases with yield close to the theoretical Thauer limit of four moles of H_2 per mole of consumed sugar (d'Ippolito et al. 2010; Pradhan et al. 2015, 2016). *T. neapolitana* also shows a novel, anaplerotic process named capnophilic lactic fermentation (CLF) that leads to the synthesis of lactic acid (LA) without affecting H_2 production under CO_2 trigger (Di Pasquale et al. 2014; d'Ippolito et al. 2014; Pradhan et al. 2017).

The ORF CTN1690 of *T. neapolitana* encodes a putative 174-aa polypeptide, with a calculated molecular weight of 19.9 kDa, and a clear homology to the O^6-alkylguanine-DNA-alkyl-transferases. Furthermore, lyophilised *T. neapolitana* cells incubated with the BG-FL substrate showed a strong fluorescent signal close to that of *Ss*OGT by gel imaging after SDS-PAGE (Fig. 2a). The observed molecular weight and, mostly the sensitivity to the benzyl-guanine derivative BG-FL substrate, led to the cloning and the heterologous expression in *E. coli* of the His-tagged version of this protein. After purification by affinity chromatography, the protein was fully active on BG-FL (Fig. 2a) and was further subjected to a biochemical characterization. The inhibition assay in the presence of methylated-dsDNA (Table 2) and the fluorescent substrate BG-FL allowed the determination of an IC_{50} value similar to that obtained with the *S. solfataricus* enzyme, thus confirming a role in DNA repair of this thermophilic





protein, hereinafter properly named *Tn*OGT (Table 2; Perugino et al. 2015). Surprisingly, the enzyme from *T. neapolitana* displayed a very high activity at low temperatures (Table 3), similar to that shown by the *Ss*OGT-H^5 mutant (Perugino et al. 2012; Vettone et al. 2016). This feature hampered the determination of second order constants at temperatures above 50.0 °C, since its reaction rate went beyond the technical limits of our assay. Such a high activity at moderate temperatures was obtained with the H^5 mutant by replacing the conserved S132 with

◂**Fig. 2** The OGT from *Thermotoga neapolitana*. **a** Lyophilized *T. neapolitana* cells grown in the presence of CO_2, were resuspended in PBS 1× buffer and BG-FL 5.0 µM and incubated 120 min at the indicated temperatures; NT, resuspended cells immediately loaded on SDS-PAGE. The *Tn*OGT gene was expressed in *E. coli* and protein was purified by His-tag affinity chromatography, as descripted in the "Materials and methods". Lane M: molecular weight marker; lane 1: cell-free extract; lane 2: flowthrough; lanes 3 and 4: column washing; lanes 5–8: eluted protein by imidazole gradient. Filled and empty green arrows indicate labelled proteins and the BG-FL substrate, respectively. **b** Superimposition between the *Ss*OGT 3D structure (PDB ID: 4ZYE; coloured as described in the legend) and a model of the *Tn*OGT (in gray). Insets represent a zoom-view of local ionic interactions in *Ss*OGT compared with the same positions in *Tn*OGT. Atoms are coloured according the CPK convention (carbon, in the corresponding colour of each 3D structure; nitrogen, in blue; oxygen, in red; sulphur, in yellow)

a glutamic acid residue. This replacement was also performed on the SNAP-tag®: the substitution led to a strong enhancement of the activity of both these engineered OGTs towards the O^6-BG derivative substrates (Juillerat et al. 2003; Perugino et al. 2012). However, the superimposition analysis between a *Tn*OGT model (constructed by the i-TASSER freeware; https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the 3D structure of the free form of *Ss*OGT (PDB ID: 4ZYE) revealed the presence of the conserved serine in *Tn*OGT (Fig. 2b): evidently, other residues in the active site contribute to the exceptional catalytic activity of this enzyme.

On the other hand, *Tn*OGT was unusually prone to degradation during the storage at − 20.0 °C, showing a series of bands below the full length, both in fluorescence imaging and in coomassie staining (Fig. S1a). This lability hampered measurements of thermostability from the DSF analysis. The reason for this degradation is unexpected: the recombinant enzyme is in its full-length form in *E. coli* cells, as well as after heat treatment of the cell free extract, and finally after purification by affinity chromatography (see "Materials and methods"; Fig. 2a). From coomassie staining analysis, three main bands of approx. 17.5, 16.1 and 14.7 kDa were detected (Fig. S1). Since they were also fluorescent (and, therefore, catalytically active), it is probable that these cuts are at the expense of the N-terminal domain, still keeping the polypeptides joined together, and making the enzyme catalytically active in solution. Again, from the superimposition, in *Tn*OGT some residues that play an important role in the stabilization of *Ss*OGT are missing. In particular, the ionic interactions that have a crucial role in the stability of the *Saccharolobus* enzyme at high temperatures, as the R133-D27 pair (Perugino et al. 2015) and the K-48 network (Morrone et al. 2017), are mainly replaced by hydrophobic residues in the *Thermotoga* homolog.

The *Pyrococcus furiosus* O^6-alkylguanine-DNA-alkyl-transferase

Pyrococcus furiosus is one of the best-studied representatives among microorganisms able to thrive above the boiling point of water (Kengen 2017). It was originally isolated anaerobically from geothermally heated marine sediments of Porto Levante (Vulcano Island, Italy), and described in 1986 by Karl Stetter and Gerhard Fiala (Fiala and Stetter 1986). This microorganism in the last decades was a source of thermostable enzymes, with potential applications in various industrial processes: the most famous example is the DNA polymerase I described in 1991 (Lundberg et al. 1991), possessing an associated 3′–5′ exonuclease activity (Kengen 2017). Recently, the discovery of the CRISPR-Cas systems in *P. furiosus* provides fundamental knowledge for new biomedical and biotechnological applications (Hael et al. 2009; Terns and Terns 2013).

In 1998, Margison and co-workers demonstrated the presence of an OGT activity in *P. furiosus*, identifying a 22.0 kDa size band by an SDS-PAGE fluorography assay

Table 2 DNA repair activity of OGTs by competitive inhibition studies in the presence of BG-FL (used as substrate) and a ds oligonucleotide containing an O^6-methylated-guanine (as inhibitor; see Table 1)

	IC_{50} (µM)	Notes
*Ss*OGT	1.01 ± 0.08	From Perugino et al. (2015)
*Tn*OGT	0.53 ± 0.13	This study
*Pfu*OGT	0.88 ± 0.10[a]	This study

[a]Performed at 65 °C

Table 3 Catalytic activities as a function of temperature of thermostable OGTs, expressed as second-order rate constant values in the presence of the sole BG-FL substrate

	T (°C)	*K* (s^{-1} M^{-1})	Notes
*Ss*OGT	25	2.80×10^3	From Perugino et al. (2012)
	50	1.50×10^4	This study
	70	5.33×10^4	From Perugino et al. (2012)
	80	ND	This study
*Tn*OGT	25	4.65×10^4	This study
	50	2.19×10^4	This study
	70	ND	This study
	80	ND	This study
*Pfu*OGT	25	ND	This study
	50	1.80×10^3	This study
	70	2.30×10^3	This study
	80	1.20×10^4	This study
	90	1.50×10^5	This study

ND not determined

(Skorvaga et al. 1998). Furthermore, this activity was completely abolished by the treatment with the O^6-benzylguanine (O^6-BG) inhibitor. This information opened the possibility of employing a hyper-thermostable enzyme as SNAP-tag® in in vivo CRISPR-Cas system-based applications. The ORF PF1878 is relative to a 174-aa polypeptide, with an expected m.w. of 20.1 kDa: the primary structure is closed to that from the MGMT from *Thermococcus kodakarensis* (*Tk*-MGMT), a well-known enzyme, in terms of structure and thermal stability (Fig. 3) (Leclere et al. 1998; Hashimoto et al. 1999; Nishikori et al. 2005). T*k*-MGMT is a very thermostable enzyme, and from its solved 3D structure (PDB ID: 1MGT) emerges that a lot of intra-helix ion-pairs contribute to reinforce stability of α-helices, whereas the presence of inter-helix ion-pairs stabilize internal packing of tertiary structure (Hashimoto et al. 1999).

The cloning of the ORF PF1878 and the subsequent expression and purification of the relative protein allowed to a complete characterization of this new enzyme. Likewise *Tn*OGT, the enzyme from *P. furiosus* is fully active on BG-FL substrate and displayed a clear ability to repair methylated DNA, as shown in the IC_{50} experiment listed in Table 2. On the other hand, *Pfu*OGT is a strong thermophilic enzyme, displaying a measurable catalytic activity only at very high temperatures (Table 4), whereas at moderate temperatures the rate of the reaction is slow, making difficult to perform the fluorescent assay. For this reason, the competitive inhibition in the presence of methylated DNA was performed at 65.0 °C instead of the standard procedure at 50.0 °C (Perugino et al. 2015; Morrone et la. 2017): however, at this temperature, the activity of *Pfu*OGT on single- and/or double-methylated DNA cannot be excluded.

The stability of this enzyme was compared with that of *Ss*OGT by the Differential Scan Fluorimetry analysis: the latter was previously treated using a scan rate of 5 min/°C × cycle (Perugino et al. 2015; Vettone et al. 2016) instead of the classical 1 min/°C × cycle (Niesen et al. 2007). Due to its very high thermal stability, a further increase of the time (10 min/°C × cycle) was necessary for *Pfu*OGT, to obtain the sigmoidal curve to fit with the Boltzmann equation (Niesen et al. 2007). In these new conditions, the T_m value of *Ss*OGT drops by approx. 13.0 °C (67.9 ± 1.1; Table 4), whereas *Pfu*OGT displayed a T_m value over 80.0 °C. The stability of *Pfu*OGT was also tested in the presence of perturbants, as high ionic strength or detergents. In the first case, we tested the importance of ionic interactions involved in the maintenance of the structure: as expected, the presence of 4.0 M NaCl strongly affected the stability of *Pfu*OGT, whereas *Ss*OGT has even shown an increase of the T_m value. Nevertheless, *Ss*OGT is more sensitive to the SDS,

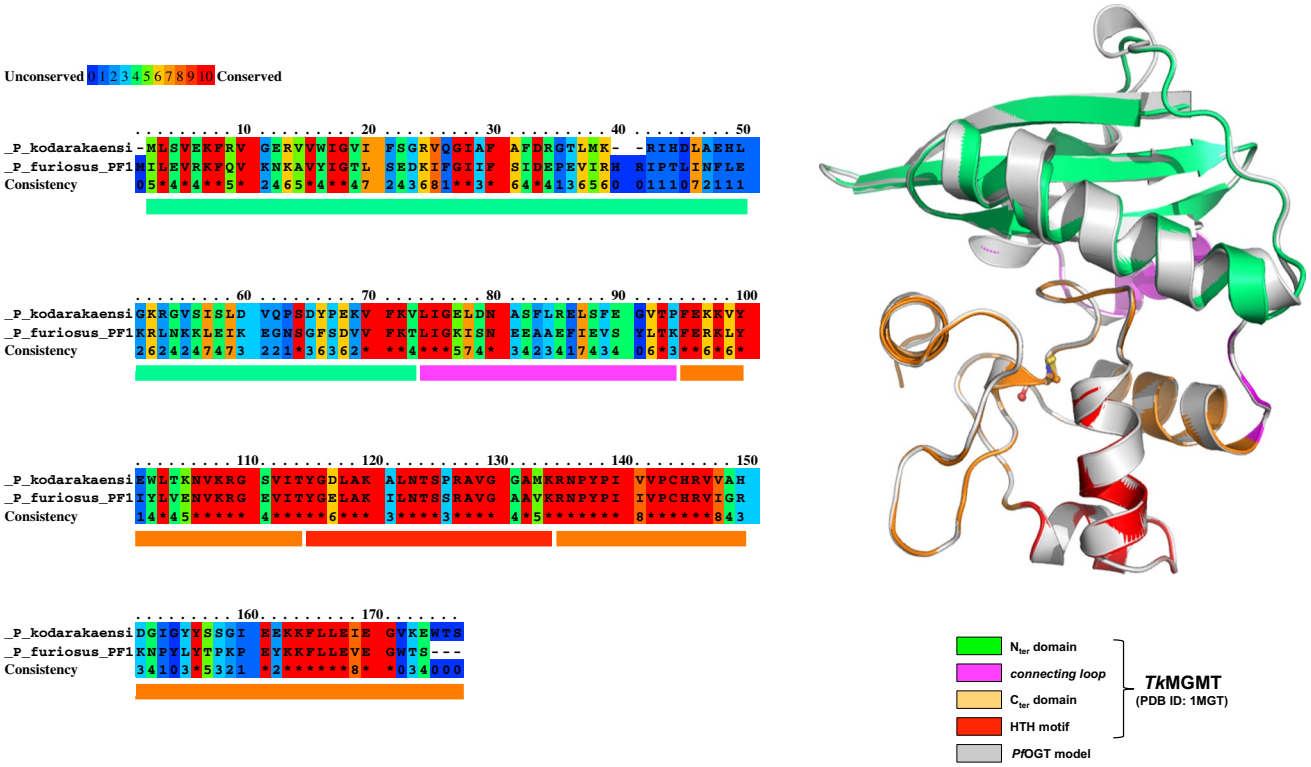


Fig. 3 Primary structure alignment and superimposition between the *Pfu*OGT and the OGT from *T. kodakarensis* (*Tk*-MGMT; PDB ID: 1MGT). Conservation of residues and protein domains are coloured on the basis of the respective legends

Table 4 Protein thermal stability by DSF method.

	Conditions	T_m (°C)	Rate (min/°C × cycle)	Notes
*Ss*OGT	PBS 1×	80.0 ± 0.4	5	From Perugino et al. (2015)
	PBS 1×	67.9 ± 1.1	10	This study
	PBS 1×; NaCl 1.0 M	79.6 ± 0.3	10	This study
	PBS 1×; NaCl 4.0 M	82.9 ± 0.4	10	This study
	PBS 1×; SDS 0.01%	46.5 ± 1.7	10	This study
*Pfu*OGT	PBS 1×	78.8 ± 0.4	10	This study
	PBS 1×; NaCl 1.0 M	83.7 ± 0.3	10	This study
	PBS 1×; NaCl 4.0 M	50.4 ± 2.1	10	This study
	PBS 1×; SDS 0.01%	71.8 ± 0.3	10	This study

T_m values were obtained by plotting the relative fluorescence intensity as a function of temperature. Data were achieved from three independent experiments

showing a collapsed T_m value up to 22.0 °C, while *Pfu*OGT drops by about 10.0 °C. Taken together, the data obtained clearly indicate a difference in the strategies of protein stabilization adopted by these two enzymes (Table 4).

Conclusion and perspectives

The modification of AGTs to produce new protein-tags for use in the "SNAP-tag® technology" offers a lot of advantages for the specific labelling of a protein of interest with an innumerable number of chemical groups conjugated to a classical inhibitor of this class of enzymes, the O^6-BG (Fig. 1b) (Hinner and Johnsson 2010). A further step forward was to adapt to this new biotechnology to a thermostable OGT from a microorganism that lives at high temperatures. After the production of *Ss*OGT-H^5 mutant, here we propose two new thermostable OGTs, which will be modified in the future to abolish their ability to bind DNA, without, however, decreasing their activity and stability to heat and to denaturing agents. The new protein-tags can be used in *T. neapolitana* and *P. furiosus*, to analyze in vivo the functions of proteins and enzymes of interest in these model systems.

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References

Aliye N, Fabbretti A, Lupidi G, Tsekoa T, Spurio R (2015) Engineering color variants of green fluorescent protein (GFP) for thermostability, pH-sensitivity, and improved folding kinetics. Appl Microbiol Biotechnol 99:1205–1216

Aoki K, Natsume A (2019) Overview of DNA methylation in adult diffuse gliomas. Brain Tumor Pathol 36:84–91

Ashby MC, Ibaraki K, Henley JM (2004) It's green outside: tracking cell surface proteins with pH-sensitive GFP. Trends Neurosci 27:257–261

Belkin S, Wirsen CO, Jannasch HW (1986) A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. App Environ Microbiol 51:1180–1185

Campbell TN, Choy FYM (2000) The effect of pH on green fluorescent protein: a brief review. Mol Biol Today 2:1–4

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 5148:802–805

Conners SB, Mongodin EF, Johnson MR, MonteroCI Nelson KE, Kelly RM (2006) Microbial biochemistry, physiology, and biotechnology of hyperthermophilic *Thermotoga* species. FEMS Microb Rev 30:872–905

Coulter R, Blandino M, Tomlinson JM, Pauly GT, Krajewska M, Moschel RC, Peterson LA, Pegg AE, Spratt TE (2007) Differences in the rate of repair of O^6-alkylguanines in different sequence contexts by O^6-alkylguanine-DNA alkyltransferase. Chem Res Toxicol 20:1966–1971

d'Ippolito G, Dipasquale L, Vella FM, Romano I, Gambacorta A, Cutignano A, Fontana A (2010) Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. Int J Hydrog En 35:2290–2295

d'Ippolito G, Dipasquale L, Fontana A (2014) Recycling of carbon dioxide and acetate as lactic acid by the hydrogen-producing bacterium *Thermotoga neapolitana*. Chemsuschem 7:2678–2683

Daniels DS, Mol CD, Arvai AS, Kanugula S, Pegg AE, Tainer JA (2000) Active and alkylated human AGT structures: a novel zinc site, inhibitor and extrahelical base binding. EMBO J 19:1719–1730

Daniels DS, Woo TT, Luu KX, Noll DM, Clarke ND, Pegg AE, Tainer JA (2004) DNA binding and nucleotide flipping by the human DNA repair protein AGT. Nat Struct Mol Biol 11:714–720

Del Prete S, Merlo R, Valenti A, Mattossovich R, Rossi M, Carginale M, Supuran CT, Perugino G, Capasso C (2019) Thermostability enhancement of the α-carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* by using the anchoring-and-self-labelling-protein-tag system (ASL^{tag}). J Enz Inhib Med Chem 34:946–954

Dipasquale L, d'Ippolito G, Fontana A (2014) Capnophilic lactic fermentation and hydrogen synthesis by *Thermotoga neapolitana*: an unexpected deviation from the dark fermentation model. Int J Hydrog En 39:4857–4862

Donaldson T, Iozzino L, Deacon LJ, Billones H, Ausili A, D'Auria S, Dattelbaum JD (2017) Engineering a switch-based biosensor for arginine using a *Thermotoga maritima* periplasmic binding protein. An Biochem 525:60–66

Fang Q, Kanugula S, Pegg AE (2005) Function of domains of human O^6-alkyl-guanine-DNA alkyltransferase. Biochemistry 44:15396–15405

Fiala G, Stetter KO (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100 °C. Arch Microb 145:56–61

Fink M, Trunk S, Hall M, Schwab H, Steiner K (2016) Engineering of TM1459 from *Thermotoga maritima* for increased oxidative alkene cleavage activity. Front Microb. https://doi.org/10.3389/fmicb.2016.01511

Gautier A, Juillerat A, Heinis C, Corrêa IR Jr, Kindermann M, Beaufils F, Johnsson K (2008) An engineered protein-tag for multiprotein labeling in living cells. Chem Biol 15:128–136

Gronemeyer T, Chidley C, Juillerat A, Heinis C, Johnsson K (2006) Directed evolution of O^6-alkylguanine-DNA alkyltransferase for applications in protein labeling. Prot Eng Des Sel 19:309–316

Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, Terns RM, Terns MP (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 139:945–956

Han D, Xu Z (2017) Development of a pyrE-based selective system for *Thermotoga* sp. strain RQ7. Extremophiles 21:297–306

Hashimoto H, Inouel T, Nishioka M, Fujiwara S, Takagi M, Imanaka T, Kai Y (1999) Hyperthermostable protein structure maintained by intra and inter-helix ion-pairs in archaeal 06-methylguanine-DNA methyltransferase. J Mol Biol 292:707–716

Hinner MJ, Johnsson K (2010) How to obtain labeled proteins and what to do with them. Curr Opin Biotechnol 21:766–776

Ishiguro K, Shyam K, Penketh PG, Sartorelli AC (2008) Development of an O^6-alkyl-guanine-DNA alkyltransferase assay based on covalent transfer of the benzyl moiety from [benzene-3H]O^6-benzylguanine to the protein. Anal Biochem 383:44–51

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch Microbiol 150:103–104

Juillerat A, Gronemeyer T, Keppler A, Gendreizig S, Pick H, Vogel H, Johnsson K (2003) Directed evolution of O^6-alkylguanine-DNA alkyltransferase for efficient labeling of fusion proteins with small molecules in vivo. Chem Biol 10(4):313–317

Kengen SWM (2017) *Pyrococcus furiosus*, 30 years on. Microb Biotechnol 10:1441–1444

Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel Johnsson K (2003) A general method for the covalent labeling of fusion proteins with small molecules in vivo. Nat Biotechnol 21:86–89

Keppler A, Pick H, Arrivoli C, Vogel H, Johnsson K (2004) Labeling of fusion proteins with synthetic fluorophores in live cells. Proc Natl Acad Sci U S A 10:9955–9959

Leclere MM, Nishioka M, Yuasa T, Fujiwara S, Takagi M, Imanaka T (1998) The O^6-methylguanine-DNA methyltransferase from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1: a thermostable repair enzyme. Mol Gen Genet 258:69–77

Lo-Gullo G, Mattossovich R, Perugino G, La Teana A, Londei P, Benelli D (2019) Optimization of an in vitro transcription/translation system based on *Sulfolobus solfataricus* cell lysate. Archaea 1:1–10

Lundberg KS, Shoemaker DD, Adams MWW, Short JM, Sorge JA, Mathur EJ (1991) High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. Gene 108:1–6

Luu KX, Kanugula S, Pegg AE, Pauly GT, Moschel RC (2002) Repair of oligodeoxyribonucleotides by O^6-alkylguanine-DNA alkyltransferase. Biochemistry 41:8689–8697

Merlo R, Del Prete S, Valenti A, Mattossovich R, Carginale V, Supuran CT, Capasso C, Perugino G (2019) An AGT-based protein-tag system for the labelling and surface immobilization of enzymes on *E. coli* outer membrane. J Enz Inhib Med Chem 34:490–499

Miggiano R, Casazza V, Garavaglia S, Ciaramella M, Perugino G, Rizzi M, Rossi F (2013) Biochemical and structural studies of the *Mycobacterium tuberculosis* O^6-methylguanine methyltransferase and mutated variants. J Bacteriol 195:2728–2736

Miggiano R, Valenti A, Rossi F, Rizzi M, Perugino G, Ciaramella M (2017) Every OGT is illuminated … by fluorescent and synchrotron lights. Int J Mol Sci 18:2613–2631

Mishina Y, Duguid EM, He C (2006) Direct reversal of DNA alkylation damage. Chem Rev 106:215–232

Mollwitz B, Brunk E, Schmitt S, Pojer F, Bannwarth M, Schiltz M, Rothlisberger U, Johnsson K (2012) Directed evolution of the suicide protein O^6-alkylguanine-DNA alkyltransferase for Increased reactivity results in an alkylated protein with exceptional stability. Biochemistry 51:986–994

Morrone C, Miggiano R, Serpe M, Massarotti A, Valenti A, del Monaco G, Rossi M, Rossi F, Rizzi M, Perugino G, Ciaramella M (2017) Interdomain interactions rearrangements control the reaction steps of a thermostable DNA alkyltransferase. Biochem Biophys Acta 1861:86–96

Niesen FH, Berglund H, Vedadi M (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat Protoc 2:2212–2221

Nishikori S, Shiraki K, Fujiwara S, Imanaka T, Takagi M (2005) Unfolding mechanism of a hyperthermophilic protein O^6-methylguanine-DNA methyltransferase. Biophys Chem 116:97–104

Pegg AE (2011) Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools. Chem Res Toxicol 24:618–639

Pegg AE, Goodtzova K, Loktionova NA, Kanugula S, Pauly GT, Moschel RC (2001) Inactivation of human O^6-alkylguanine-DNA alkyltransferase by modified oligodeoxyribonucleotides containing O^6-benzylguanine. J Pharmacol Exp Ther 296:958–965

Perugino G, Vettone V, Illiano G, Valenti A, Ferrara MC, Rossi M, Ciaramella M (2012) Activity and regulation of archaeal DNA alkyltransferase: conserved protein involved in repair of DNA alkylation damage. J Biol Chem 287:4222–4231

Perugino G, Miggiano R, Serpe M, Vettone A, Valenti A, Lahiri S, Rossi F, Rossi M, Rizzi M, Ciaramella M (2015) Structure-function relationships governing activity and stability of a DNA alkylation damage repair thermostable protein. Nucleic Acids Res 43:8801–8816

Pradhan N, Dipasquale L, d'Ippolito G, Panico A, Lens PNL, Esposito G, Fontana A (2015) Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Int J Mol Sci 16:12578–12600

Pradhan N, Dipasquale L, d'Ippolito G, Fontana A, Panico A, Lens PNL, Pirozzi F, Esposito G (2016) Kinetic modeling of fermentative hydrogen production by *Thermotoga neapolitana*. Int J Hydrog Energy 41:4931–4940

Pradhan N, Dipasquale L, d'Ippolito G, Panico A, Lens PNL, Esposito G, Fontana A (4359T) Hydrogen and lactic acid synthesis by the wild-type and a laboratory strain of the hyperthermophilic bacterium *Thermotoga neapolitana* DSMZ 4359T under capnophilic lactic fermentation conditions. Int J Hydrog Energy 42:16023–16030

Samuelson P, Gunneriusson E, Nygren PA, Ståhl S (2002) Display of proteins on bacteria. J Biotechnol 96:129–154

Serpe M, Forenza C, Adamo A, Russo N, Perugino G, Ciaramella M, Valenti A (2019) The DNA Alkylguanine DNA

Alkyltransferase-2 (AGT-2) of *Caenorhabditis Elegans* is involved in meiosis and early development under physiological conditions. Sci Rep 9:6889

Skorvaga M, Raven NDH, Margison GP (1998) Thermostable archaeal O^6-alkylguanine-DNA alkyltransferases. Proc Natl Acad Sci USA 95:6711–6715

Sun G, Zhao L, Zhong R, Peng Y (2018) The specific role of O^6-methylguanine-DNA methyltransferase inhibitors in cancer chemotherapy. Future Med Chem 10:1971–2199

Terns RM, Terns MP (2013) The RNA- and DNA-targeting CRISP-Cas immune systems of *Pyrococcus furiosus*. Biochem Soc Trans 41:1416–1421

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67:509–544

Tubbs JL, Pegg AE, Tainer JA (2007) DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O^6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. DNA Repair 6:1100–1115

Vettone A, Serpe M, Hidalgo A, Berenguer J, del Monaco G, Valenti A, Rossi M, Ciaramella M, Perugino G (2016) A novel thermostable protein-tag: optimization of the *Sulfolobus solfataricus* DNA-alkyl-transferase by protein engineering. Extremophiles 20:1–13

Visone V, Han W, Perugino G, del Monaco G, She Q, Rossi M, Valenti A, Ciaramella M (2017) In vivo and in vitro protein imaging in thermophilic archaea by exploiting a novel protein tag. PLoS ONE 12:e0185791

Yang CG, Garcia K, He C (2009) Damage detection and base flipping in direct DNA alkylation repair. ChemBioChem 10:417–423

Zhang J, Shi H, Xu L, Zhu X, Li X (2015) Site-directed mutagenesis of a hyperthermophilic endoglucanase Cel12B from *Thermotoga maritima* based on rational design. PLoS ONE. https://doi.org/10.1371/journal.pone.0133824