

# Functional and structural characterization of protein disulfide oxidoreductase from *Thermus thermophilus* HB27

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**Abstract** The paper reports the characterization of a protein disulfide oxidoreductase (PDO) from the thermophilic Gram negative bacterium *Thermus thermophilus* HB27, identified as TTC0486 by genome analysis and named *Tt*PDO. PDO members are involved in the oxidative folding, redox balance and detoxification of peroxides in thermophilic prokaryotes. *Ttpdo* was cloned and expressed in *E. coli* and the recombinant purified protein was assayed for the dithiol-reductase activity using insulin as substrate and compared with other PDOs characterized so far. In the thermophilic archaeon *Sulfolobus solfataricus* PDOs work as thiol-reductases constituting a peculiar redox couple with Thioredoxin reductase (SsTr). To get insight into the role of *Tt*PDO, a hybrid redox couple with SsTr, homologous to putative Trs of *T. thermophilus*, was assayed. The results showed that SsTr was able to reduce *Tt*PDO in a concentration dependent manner with a calculated  $K_M$  of 34.72  $\mu$ M, suggesting the existence of a new redox system

also in thermophilic bacteria. In addition, structural characterization of *Tt*PDO by light scattering and circular dichroism revealed the monomeric structure and the high thermostability of the protein. The analysis of the genomic environment suggested a possible clustering of *Ttpdo* with TTC0487 and TTC0488 (*tlpA*). Accordingly, transcriptional analysis showed that *Ttpdo* is transcribed as polycistronic messenger. Primer extension analysis allowed the determination of its 5' end and the identification of the promoter region.

**Keywords** Protein disulfide oxidoreductase · Oxidative folding · Thermophilic microorganisms · *Thermus thermophilus*

## Introduction

Oxidative protein folding, that involves the formation and isomerization of disulfide bridges, plays a key role in the stability of proteins and is essential for many regulatory functions as well as redox homeostasis. In all the three domains of life the formation and the breakage of disulfide bridges is generally catalyzed by thiol-disulfide oxidoreductases. These enzymes are characterized by one or more Thioredoxin (Trx) folds that consist of a four-stranded  $\beta$ -sheet surrounded by three  $\alpha$ -helices, with a CXXC redox active-site motif (Berndt et al. 2008; Pedone et al. 2010). The assembly of various Trx modules has been used to build the different thiol oxidoreductases found in prokaryotic and in eukaryotic organisms. In detail, in the bacterial periplasm, the proteins are kept in the appropriate oxidation state by a combined action of the couples Disulfide bond forming proteins (Dsb) DsbB-DsbA and DsbD-DsbC/DsbE/DsbG (Inaba 2009; Hatahet et al. 2014).

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In the eukaryotes, a similar function is carried out in the endoplasmic reticulum (RE) by the protein disulfide isomerase (PDI) or other PDI-like proteins (Pedone et al. 2010; Sato and Inaba 2012; Gruber et al. 2006). PDI is characterized by two catalytic domains, a and a', each containing the CXXC motif, separated by two non-catalytic domains b and b' and a highly acidic extension at the C-terminus, named c, bearing the motif for RE-localization. In the PDI from yeast the domain a functions mainly as an isomerase, while a' presents mostly an oxidative activity (Pedone et al. 2010; Ruddon and Bedows 1997; Hatahet et al. 2014). The recycling of PDI is performed by ER oxidoreductin FAD dependent proteins as Ero1-La and Ero1-Lb in mammalian cells (Pagani et al. 2000; Cabibbo et al. 2000) and Ero1p (Gross et al. 2004, 2006; Sevier and Kaiser 2006) or Erv2p oxidase in yeast (Sevier et al. 2001; Vala et al. 2005; Gross et al. 2002). These oxido-reductase coupled reactions lead to the formation of native Dsbs in the substrate proteins.

In thermophilic archaea and bacteria, which are characterized by proteins with a high disulfide-content, oxidative protein folding in the cytoplasm has been mainly ascribed to a peculiar class of thiol-disulfide oxidoreductases named protein disulfide oxidoreductases (PDOs) (Pedone et al. 2004, 2006b, 2010; Beeby et al. 2005; Hatahet et al. 2014).

Protein disulfide oxidoreductases exclusively occur in thermophiles and in fact it has been proposed their crucial role in the adaptation to extreme conditions. Differently from PDI, the PDOs present a simpler organization with only two Trx folds, each with a CXXC motif. Four archaeal members of this family have been structurally and functionally characterized i.e. *PfpDO* from *Pyrococcus furiosus* (Guagliardi et al. 1995; Ren et al. 1998; Pedone et al. 2004), *PhPDO* from *P. horikoshii* (Kashima and Ishikawa 2003), *ApPDO* from *Aeropyrum pernix* (D'Ambrosio et al. 2006) and *SsPDO* from *Sulfolobus solfataricus* (Pedone et al. 2006b). *SsPDO*, in addition to reductase and chaperone activities, is involved in a cell defense mechanism against ROS accumulation through the reduction of peroxides (Limauro et al. 2009; Lu and Holmgren 2013). As reported by Limauro et al. 2008, *SsPDO* is the joining link from NADPH/Thioredoxin reductase (Tr) to Bacterio migratory protein (Bcp)1 or Bcp4 involved in peroxide reduction. Until today only one bacterial PDO from the hyperthermophilic *Aquifex aeolicus* (*AaPDO*) has been characterized (Pedone et al. 2006a). *AaPDO* is able to reduce, oxidize and isomerize disulfide bridges representing the first example of a bacterial disulfide isomerase not belonging to the Dsb family. It has been proposed that it could constitute a different redox system working coupled with a putative DsbC protein whose gene sequence was found in the genome (Pedone et al. 2006a). Recently in

*Thermotoga maritima* a Trx (TM0868), that shows 42 % identity with *AaPDO*, forms a redox couple with Tr (TM0869) (Yang and Ma 2010).

In this study, the Gram negative bacterium *Thermus thermophilus* was chosen to further investigate on thiol-disulfide oxidoreductase proteins in thermophilic bacteria. It grows aerobically at temperatures ranging from 50 to 82 °C, exhibits a high competence for natural transformation and therefore it is prone to easy genetic manipulations. Furthermore the genome has been completely sequenced (Genome accession number AE017221) (Henne et al. 2004) but scarce information is available on its thiol-redox systems. Therefore *T. thermophilus* shows many features that make it an intriguing model system both for basic research and for biotechnological applications (Cava et al. 2009).

In this paper we report a functional–structural characterization of a new PDO, *TtpDO*, identified on the genome of *T. thermophilus* HB27 as TTC0486.

*Ttpdo* was cloned in pET28b(+) vector and expressed in *E. coli*; the recombinant protein was purified to homogeneity and characterized for the structural and thermostability properties by light scattering and circular dichroism, respectively. *TtpDO* functional characterization was studied by measuring its reductase activity on insulin and in a hybrid redox system with Tr from *S. solfataricus* *SsTr* (Sso2476).

STRING and genomic environment analyses suggested that *Ttpdo* was clustered in an operon with TTC0487 and *tlpA* (TTC0488), encoding a putative transferase/hydrolase and a putative thiol-disulfide interchange protein, respectively. Transcriptional analysis confirmed the bioinformatics and primer extension was performed to characterize the promoter region.

## Materials and methods

### Bacterial strains and growth conditions

*T. thermophilus* HB27 strain was purchased from DSMZ. A frozen (−80 °C) stock of *T. thermophilus* HB27 was streaked on a *Thermus* Medium (TM) solidified by the addition of 0.8 % Gelrite and incubated at 74 °C overnight. A single colony was inoculated into TM liquid medium and grown as already described (Del Giudice et al. 2013).

*E. coli* strains were grown in Luria–Bertani medium at 37 °C with a 100 µg/ml ampicillin, 50 µg/ml kanamycin, tetracycline 15, 33 µg/ml chloramphenicol as required. *E. coli* *One Shot*<sup>®</sup> *TOP10* (Invitrogen) and *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA, USA) were used for DNA manipulations and for the expression of the recombinant *TtpDO*, respectively.

## Design, expression and purification of *Tt*PDO

Genomic DNA of *T. thermophilus* HB27 was prepared as already described (Del Giudice et al. 2013). The gene encoding putative *Ttpdo* (*TTC0486*) was amplified by PCR, using DyNAzyme™ EXT Taq (FINNZYMES) and the following oligonucleotides: *TTC0486-F* 5'AGGTGAC CATGGCGCTTTT-3' and *TTC0486-R* 5'-GTCAAGCT TGGCCCGGAC-3' containing the *NcoI* and *HindIII* restriction sites, respectively (underlined). The amplification was carried out at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, for 35 cycles. The PCR product were purified with QIAquick PCR purification kit (Qiagen Spa, Milan, Italy) and cloned in pCR™4-TOPO® vector using—*TOPO TA CLONING*® Kit (Invitrogen). The identity of the cloned fragment was confirmed by DNA sequencing. Then, the purified *NcoI-HindIII* fragment was cloned into pET28b(+) (Novagen, Darmstadt, Germany) digested with the same enzymes. The recombinant plasmid pET-*Tt*PDO obtained was used to transform competent *E. coli* BL21-Codon-Plus (DE3)-RIL cells. Transformed cells were grown in LB medium containing kanamycin (50 µg/ml) and chloramphenicol (33 µg/ml) up to 0.7 A<sub>600nm</sub> at 37 °C and then induced for 3 h with 0.5 mM isopropyl 1-thio-β-D galactopyranoside (Inalco S.P.A., Milan, Italy).

A pellet from 1000 ml culture was suspended in 20 ml of lysis buffer (50 mM Tris HCl pH 8.0/1 mM phenylmethylsulfonyl fluoride). The cells were homogenized by sonication using 20 min pulses at 20 Hz (Sonicator Ultrasonic liquid processor; Heat System Ultrasonics Inc., NY, USA). The lysate was centrifuged at 20000g for 90 min (JA25.50 rotor; Beckman). The supernatant was heated to 80 °C for 15 min, and denatured proteins were removed by centrifugation at 20000g for 30 min at 4 °C.

The cell extract was loaded onto a HisTrap HP (GE Healthcare, Healthcare Europe, GmbH, Milan, Italy) connected to an AKTA Explorer system (GE Healthcare) and equilibrated with 50 mM Tris HCl pH 8.0, 0.3 M NaCl (buffer A). The column was washed with buffer A plus 20 mM imidazole, and proteins were eluted with the same buffer A supplemented with 250 mM imidazole. The fractions were pooled, analyzed by SDS-PAGE and dialyzed against 20 mM Tris HCl pH 8.0.

## Determination of the reductase activities of *Tt*PDO

*Tt*PDO activity was measured both using insulin as substrate (Holmgren 1979) and in a coupled assay that follows the oxidation of NADPH in the presence of *SsTr* (Limauro et al. 2008).

In particular, the insulin reductase activity of recombinant *Tt*PDO (1.2 µM) was assayed according to

Holmgren's turbidimetric method with a few modifications described in Limauro et al. (2008, 2009).

To perform the Tr assay, *SsTr* was expressed and purified as already described (Limauro et al. 2009). The reaction mixture contained 0.1 M K-Phosphate pH 7.0, 2 mM EDTA, 0.05 mM FAD, 2 µM *SsTr*, 4–75 µM *Tt*PDO, 0.25 mM NADPH. The assay was carried out at 60 °C and the activity was determined measuring the decrease in absorbance at 340 nm using a molar extinction coefficient of 6.2 mM<sup>-1</sup> cm<sup>-1</sup>. Control reaction was performed without *SsTr*.

## Light scattering

Purified protein was analyzed by size exclusion chromatography connected to a MiniDAWN Treos light scattering system (Wyatt Technology) equipped with a QELS module (quasi-elastic light scattering) for mass value. 100 µg sample (1 mg/ml) was loaded on a Wyatt WTC015-S5 column (7.8 × 30 cm), equilibrated in 20 mM Tris HCl pH 8.0, 150 mM NaCl. A constant flow rate of 0.5 ml/min was applied. Data were analyzed by using Astra 5.3.4.14 software (Wyatt Technology).

## Circular dichroism spectroscopy

Far-UV CD spectra were recorded using a Jasco J-815 CD spectrometer, equipped with a Peltier-type temperature control system (model PTC-423S/15). Cells with path lengths of 0.1 cm were used and spectra recorded with a time constant of 4 s, a 1 nm bandwidth, and a scan rate of 20 nm/min; the signal was averaged over at least three scans and baseline corrected by subtraction of a buffer spectrum. Spectra were analyzed for secondary structure using CDPro software. CD measurements were carried out using 2.5 µM *Tt*PDO in 10 mM Tris HCl pH 7.0 buffer. *Tt*PDO thermal stability was studied both following the change in the CD signal at 222 nm with a scan rate of 1.0 °C/min (range of temperature from 30 to 105 °C) and recording spectra after incubation of *Tt*PDO at 70, 80 and 90 °C for 30, 60 and 120 min.

## Transcriptional analysis

### RNA extraction

Total RNA was extracted from 5 ml of *T. thermophilus* HB27 at 0.5 OD<sub>600nm</sub> using RNeasy® Mini Kit (Qiagen).

### Reverse transcription-PCR

RT-PCR reaction was carried out on 2 µg of DNaseI-treated RNAs using SuperScript III reverse transcriptase

(Invitrogen) following the manufacturer's protocol. The TTC0488-R primer: 5'-TTTCCGCCGGCTCGAGGCGG AGG-3' was used for the cDNA synthesis and the enzyme was denatured at 70 °C for 15 min. A negative control without reverse transcriptase was included to guarantee the absence of DNA contamination. PCR reactions were performed using the primer pairs: TTC0486-F: 5'-TCCG GCCATGGACGCCCTT-3' and TTC0488-R (see above), by 35 amplification cycles of 94 °C for 1 min, an annealing temperature of 58 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The products of PCR were detected by agarose gel electrophoresis.

### Primer extension analysis

Primer extension analysis was carried out with avian myeloblastosis virus reverse transcriptase (Roche) as described elsewhere (Limauro et al. 1996) using the synthetic oligonucleotide (prTtPDO) 5'-CGGTGAAGAGCAC CATCTC-3'. The primer was labelled at 5' end with T4 polynucleotide kinase and  $\gamma^{32}\text{P}$  ATP.

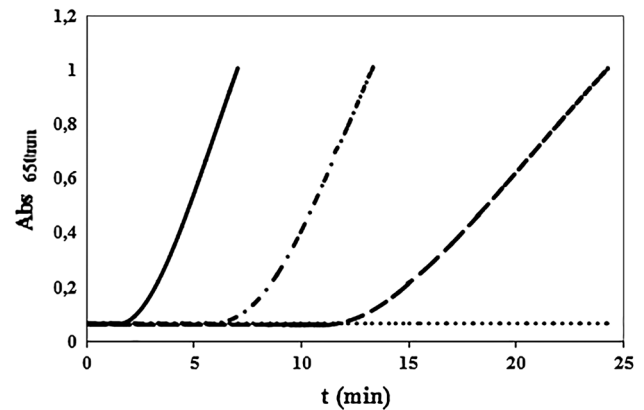
prTtPDO and seqTtPDO oligonucleotides: 5'-ATCTGG CGAAAGATCATGGC TT-3', were used to produce a sequence ladder using the "f-mol DNA Cycle Sequencing System" kit (Promega).

## Results

### Expression and purification of TtPDO

On the genome of *T. thermophilus* HB27 an ORF (TTC0486), encoding a putative glutaredoxin-like protein, was identified. The corresponding TtPDO protein has a theoretical pI of 5.35, a molecular weight of 25.4 kDa and two CXXC motifs typical of the PDO family: CLYC at the N-terminus and the more conserved sequence CPYC at the C-terminus. The BlastP analysis between TtPDO and the other characterized thermophilic PDOs shows the highest identity (37 %) with PfPDO, PhPDO and Tm0868, 33 % identity with AaPDO and ApPDO, and 28 % with SsPDO. As already reported in literature (Pedone et al. 2010), the residues around PDO active sites form two grooves, denominated N and C, which can constitute two substrate binding sites (Ren et al. 1998) While the N groove is delimited by residues belonging to the N- and C-terminal Trx units, the C groove is delimited by strictly conserved residues of the C-terminal unit, mainly hydrophobic. These residues are also conserved in the C-terminal unit of TtPDO and thus could contribute to the formation of the C groove.

TtPDO was highly overexpressed in *E. coli* in soluble form, as fusion with a C-terminal histidine tag



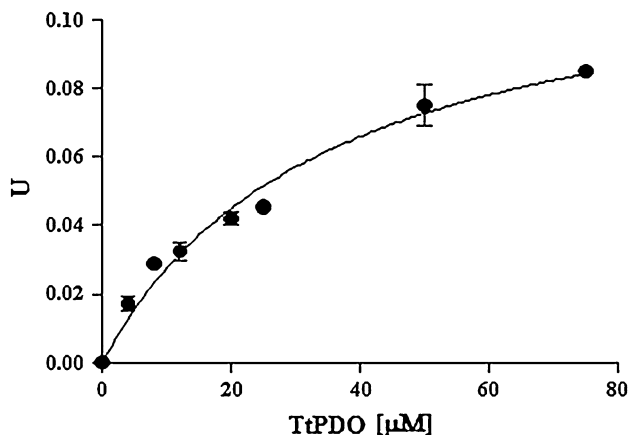
**Fig. 1** Reductase activity assay by reduction of bovine insulin disulfides. The dithiothreitol-dependent reduction of bovine insulin disulfides was carried out as described in "Materials and methods" in the absence (negative control dotted line) or presence of 1.2  $\mu\text{M}$  of pure SsPDO (dashed line), TtPDO (straight line), AaPDO (dashed with dotted line)

(LAAALEHHHHHH) in the vector pET28b(+). To purify the protein, the soluble fraction obtained after sonication was processed by heat-treatment at 80 °C for 15 min and loaded on affinity chromatography on HisTrap HP. The protein was purified to homogeneity as revealed by the single band with a molecular mass about 27 kDa showed by the SDS-PAGE (Fig. S1). The yield of the purified protein was 5 mg per liter.

### Functional characterization of TtPDO

To clarify the function of TtPDO different assays were performed. At first, in order to verify the reductase activity of TtPDO, it was tested its ability to reduce insulin disulfides in the presence of DTT as electron donor at 30 °C in the Holmgren assay (Holmgren 1979). The reduced  $\beta$ -chain of insulin precipitates causing the increase of turbidity detected at  $A_{650\text{nm}}$  demonstrating the reductase activity of the protein. In addition, comparative analysis, performed with characterized archaeal and bacterial thermophilic PDOs at the same concentration (Fig. 1), showed that TtPDO was the most active, suggesting a different catalytic activity.

Generally in thermophilic Archaea PDOs work as thiol-reductases forming a redox couple with Tr (Limauro et al. 2008, 2013), nevertheless, the role of PDOs has not yet been clarified in thermophilic bacteria. To investigate this aspect we constructed a hybrid redox system using Tr of *S. solfataricus* (SsTr) coupled with TtPDO. SsTr could be considered a good candidate because: (1) previously, it was demonstrated that SsTr works with a heterologous substrate as Trx of *E. coli* (Ruocco et al. 2004; Ruggiero et al. 2009;



**Fig. 2** Michaelis–Menten plot of *SsTr* using *TtPDO* as substrate. The activity was monitored following NADPH oxidation at  $A_{340\text{nm}}$

Del Giudice et al. 2013) constituting a hybrid redox couple; (2) BlastP analysis showed significant homology between *SsTr*, and the three putative Trs annotated on the of *T. thermophilus* genome (Henne et al. 2004): TTC0096, TTC0855 and TTC1555; (3) the analysis carried out by STRING, an interacting data base (<http://string-db.org/>), based on predicted functional parameters, showed that one of the putative Trs, TTC1555, has a high association score with *TtPDO*. Following these indications, *SsTr* reductase activity was assayed measuring the decrease of NADPH at  $A_{340\text{nm}}$  at different concentrations of *TtPDO*. The results showed that *SsTr* was able to reduce *TtPDO* in a concentration dependent manner with a calculated  $K_M$  of  $34.72 \mu\text{M}$  (Fig. 2).

#### Structural characterization of *TtPDO*

To assess the quaternary structure of the recombinant protein, gel filtration of purified *TtPDO* also coupled with light scattering methodology was performed. The results are in agreement with a monomeric structure for *TtPDO* as previously reported for other PDOs (Pedone et al. 2010). To evaluate the structural integrity and to obtain information on the secondary structure of *TtPDO*, CD spectra were registered at  $30^\circ\text{C}$  in the far-UV. The spectrum exhibits one maximum at 195 nm, a rather sharp minimum centered at about 208 nm and a broader one at 222 nm (Fig. 3a). These features are indicative of the presence of both  $\alpha$  and  $\beta$  secondary structure elements. A quantitative estimation of the secondary structure content, performed by using the software CDPPro, suggests that the  $\alpha$ -helix and the  $\beta$ -sheet contents are 35 and 22 %, respectively, in agreement with previous characterized PDOs.

The secondary structure prediction was confirmed by PSIPRED analysis (<http://bioinf.cs.ucl.ac.uk/psipred/>)

which also outlined the presence of two Trx folds, typical of members of PDO family members.

To test *TtPDO* heat stability, a thermal denaturation curve was followed at 222 nm by CD in the range  $30\text{--}105^\circ\text{C}$  (Fig. 3b). Likewise all the previously characterized PDOs, also *TtPDO* turned to be particularly stable, with a  $T_m$  of about  $97^\circ\text{C}$  (Limauro et al. 2009; Pedone et al. 2001). This value of  $T_m$  is in perfect agreement with the other characterized members of the same family which present  $T_m$  higher than  $90^\circ\text{C}$  (D'Ambrosio et al. 2006; Pedone et al. 2006a; Limauro et al. 2009). It makes exception a recently characterized atypical PDO, *Sso1120* (Limauro et al. 2013) which presents a  $T_m$  of  $90^\circ\text{C}$ . Further CD analyses performed after incubation at different times and temperatures showed a high resistance to heat (Fig. 3c), in particular after 120 min incubation at  $90^\circ\text{C}$ , it retains 50 % of its structure (Fig. 3d).

#### *Ttpdo* transcriptional analyses

Genome and nucleotide analyses in the TTC0486 genomic environment showed the presence of two other ORFs annotated as TTC0487 and TTC0488 (TlpA) that could be translationally coupled. As showed in Fig. 4a the STOP codon of TTC0486 and the ATG of TTC0487 are separated by only one nucleotide while the STOP codon of TTC0487 overlaps the ATG of TTC0488. Such genic arrangement was observed not only in other *Thermus* sp. but also in *Meiothermus silvanus* and *Oceanithermus profundus* which belong to the same family. To verify the gene clustering, RT-PCR was performed. The result showed a single transcript of about 2000 bp, indicating that TTC0486-87-88 are transcribed as a polycistronic messenger (Fig. 4b).

In order to characterize the promoter region of the operon, a primer extension analysis was carried out (Fig. 4c). As shown in Fig. 4c, the 5' end of the mRNA begins with a Timine and maps 36 nucleotides upstream of the ATG translation start codon. TCTTTCTC and TATAGT are the cis-acting regulatory sequences of the bacterial promoter centered at  $-35$  and  $-10$  nucleotides respectively from the 5' end mapped. A consensus sequence, GGAGG, resembling the Shine Dalgarno motif was observed upstream of the ATG start codon (Sanchez et al. 2000).

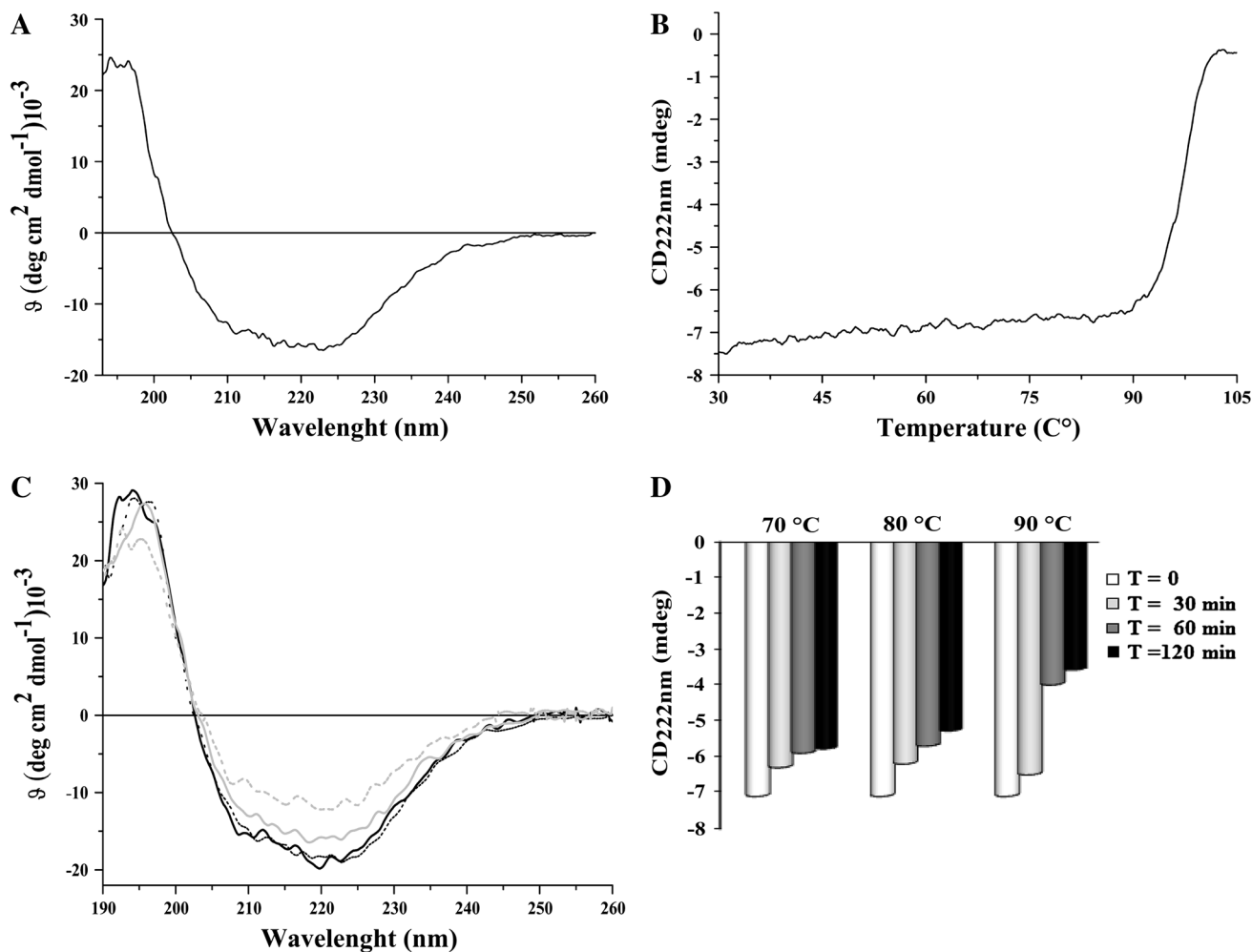
#### Discussion

In the last few years (Ladenstein and Ren 2008) the accepted dogma that the reducing state of the cytoplasm allows the oxidative metabolic reactions and defenses from the attack of the ROS, has been revised for the thermophilic microorganisms. In particular in these microorganisms biochemical

and proteomic evidences indicate a richness of proteins with Dsbs (Beeby et al. 2005), and a ratio GSSG/GSH much higher with respect to mesophilic microorganisms (Heinemann et al. 2014). The redox homeostasis inside the cell is generally guaranteed by a pool of small molecules and protein thiols, however recent genomic, proteomic and metabolomics studies support the hypothesis that in thermophilic microorganisms this breakable balance could be maintained by different systems.

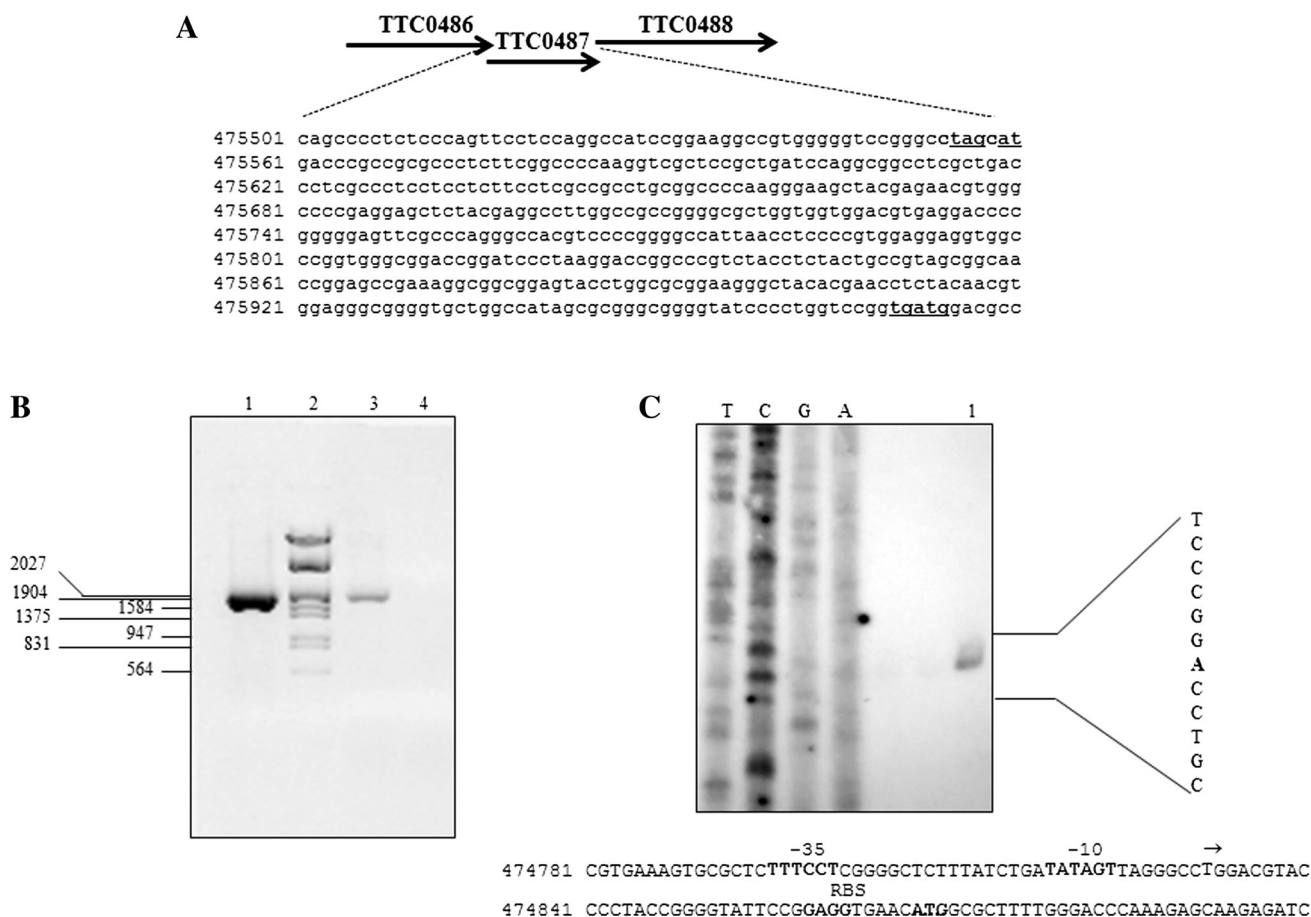
In eukaryotes and mesophilic bacteria the oxidative protein folding occurs exclusively in the ER and the periplasmic space, depending upon PDI and Dsbs respectively; in the thermophilic microorganisms a further exclusive intracellular oxido-reduction system of Dsbs ascribed to PDOs exists, opening a new scenario on the chemistry of cytoplasmic environment.

To investigate on the redox systems of thermophilic bacteria (Pedone et al. 2006a), *T. thermophilus* has been chosen to characterize a new PDO produced in recombinant form in *E. coli*. The reported results showed that *Tt*PDO has reductase activity and displays high thermostability. From a structural point of view, *Tt*PDO is a monomer with two conserved Trx folds. From a functional point of view similarly to the two PDOs from *S. solfataricus* (Limauro et al. 2009, 2013), *Tt*PDO could form a redox couple with Tr, as suggested in the generated hybrid redox system *Ss*Tr/*Tt*PDO. In fact *Ss*Tr can reduce *Tt*PDO with  $K_M$  of 34.72  $\mu$ M. In *T. thermophilus* genome there are annotated three ORFs encoding putative Trs and one of them, TTC1555, has the highest identity (36 %) with *Ss*Tr; in addition, the interacting data base STRING supports a possible link between *Tt*PDO and TTC1555.



**Fig. 3** *Tt*PDO CD analyses. **a** Far UV CD spectrum registered at 30 °C. **b** Thermal denaturation curve monitored following CD signal at 222 nm. **c** Far UV CD spectra registered after 60 min incubation at 30 °C (continuous black), 70 °C (dotted black), 80 °C (grey

continuous) and 90 °C (dotted grey). **d** Comparative analysis of the conformational stability after incubation for 0 min (white), 30 min (grey), 60 min (gun metal), 120 min (black) at 70, 80, 90 °C monitored following CD signal at 222 nm



**Fig. 4 a** Schematic representation of TTC0487 locus and overlapping coding sequences of TTC0486 and TTC0488. *Underlined* are the stop codon of TTC0486 spaced by one nucleotide from the start codon of TTC0487 and the stop codon of TTC0487 overlapping with the start codon of TTC0488. **B.** RT-PCR analysis of TTC0486-TTC0487-TTC0488. *Lane 1* PCR product amplified from *T. thermophilus* genome as positive control, *lane 2* Marker III (Roche), *lane 3*

RT-PCR product, *lane 4* negative control incubated without reverse transcriptase. **c** Primer extension analysis of *Ttpdo*. Transcriptional start point is shown by the *arrow*; reference number of *T. thermophilus* HB27 genome sequence is reported on the *left*. -35 and -10 consensus promoter regions and ribosome binding site (RBS) are in *bold*. ATG is *underlined*

Even though the function of *TtPDO* in the redox couple with the Tr TTC1555 must be still investigated, other interesting suggestions about its potential role have arisen from the transcriptional analysis. RT-PCR indicates that *Ttpdo* forms a polycistronic mRNA with TTC0487-TTC0488 of about 2000 bp, indicating a possible functional correlation with the two proteins encoding a putative hydrolase (TTC0487) and a putative thiol:disulfide interchange protein (TTC0488-TlpA), respectively. The possible functional link between *TtPDO* and putative TlpA is underlined also by the conservation of the genomic environment within the *Thermaceae* family.

Altogether, our results suggest that *TtPDO* could interact not only with Tr but also with TlpA and that could be involved in a number of different redox pathways significantly broader than it was previously thought. In addition PSORT prediction analysis indicated

a cytoplasmic membrane localization for TlpA suggesting, similarly to other Gram negative bacteria (Achard et al. 2009; Cho et al. 2012), that this protein could be involved in the antioxidant response in the periplasmic space associated with thiol reducing power from the cytoplasm. These evidences encourage further investigations on the role of the new hypothetical *TtPDO*-TlpA couple in the peroxide detoxification or in other pathways as the assembly of cytochrome c (Tanboon et al. 2009).

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