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ORIGINAL PAPER

Gene cloning and protein expression of γ-glutamyltranspeptidases from *Thermus thermophilus* and *Deinococcus radiodurans*: comparison of molecular and structural properties with mesophilic counterparts

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Abstract γ-Glutamyltranspeptidase (γ-GT) is an ubiquitous enzyme that catalyzes the hydrolysis of γ-glutamyl bonds in glutathione and glutamine and the transfer of the released γ-glutamyl group to amino acids or short peptides. γ-GTs from extremophiles, bacteria adapted to live in hostile environments, were selected as model systems to study the molecular underpinnings of their adaptation to extreme conditions and to find out special properties of potential biotechnological interest. Here, we report the cloning, expression and purification of two members of γ-GT family from two different extremophilic species, *Thermus thermophilus* (*Tt*GT) and *Deinococcus radiodurans* (*Dr*GT); the first is an aerobic eubacterium, growing at high temperatures (50–82°C), the second is a polyextremophile, as it tolerates radiations, cold, dehydration, vacuum, and acid. *Tt*GT and *Dr*GT were both synthesized as precursor proteins of 59–60 kDa, undergoing an intramolecular auto-cleavage to yield two subunits of 40 and 19–20 kDa, respectively. However, like the γ-GT from *Geobacillus thermodenitrificans*, but differently from the other characterized bacterial and eukaryotic γ-GTs, the two new extremophilic enzymes displayed γ-glutamyl hydrolase, but not transpeptidase activity in the 37–50°C temperature range, pH 8.0. The comparison of sequences and structural models of these two proteins with experimental-determined structures of other known mesophilic γ-GTs suggests that the extremophilic members of this protein family have found a common strategy to adapt to different hostile environments. Moreover, a phylogenetic analysis suggests that γ-GTs displaying only γ-glutamyl hydrolase activity could represent the progenitors of the bacterial and eukaryotic counterparts.

Keywords Gamma-glutamyltranspeptidases · Molecular adaptation · Extremophiles · *Thermus thermophilus* · *Deinococcus radiodurans*

Abbreviations

γ-GT	Gamma-glutamyltranspeptidase
Bs	*Bacillus subtilis*
Bh	*Bacillus halodurans*
Dr	*Deinococcus radiodurans*
Ec	*Escherichia coli*
Gth	*Geobacillus thermodenitrificans*
Hp	*Helicobacter pylori*
h	human
Ta	*Thermoplasma acidophilum*
Tt	*Thermus thermophilus*
GNA	L-Glutamic acid γ-(4-nitroanilide)
GSH	Glutathione

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Introduction

γ-Glutamyltranspeptidase (γ-GT) catalyzes the hydrolysis of γ-glutamyl compounds, such as glutathione (GSH, γ-glutamyl-cysteinyl-glycine) to yield glutamic acid and cysteinyl-glycine, or glutamine to yield glutamic acid and ammonia. γ-GTs can also catalyze the transfer of the

released glutamyl moieties to amino acids or short peptides (Tate and Meister 1981). These enzymes are ubiquitous proteins, found from bacteria to mammals, involved in the γ-glutamyl cycle as they catalyze the initial step of the degradation of the extracellular GSH into its constituent amino acids, which are then transported into the cell and reused in protein biosynthesis. Thanks to the property of GSH to keep the reduced intracellular environment, γ-GTs are also involved in the cell defence mechanism against oxidative stress (Pompella et al. 2007; Emdin et al. 2005). Bacterial γ-GTs are generally soluble and localized in the periplasmic space by an N-terminal signal peptide or secreted in the extracellular environment, whereas mammalian counterparts are embedded in the plasma membrane by an N-terminal trans-membrane peptide and are heterologously glycosylated. Even though taxonomically distant γ-GTs share >25% sequence identity suggesting a strong conservation of structure and function, significant catalytic differences exist between bacterial and non-bacterial counterparts (Boanca et al. 2006). γ-GT is synthesized as a precursor protein of 60 kDa, which undergoes an intra-molecular autocatalytic cleavage yielding a large (40 kDa) and a small (20 kDa) subunit (Boanca et al. 2006; Suzuki and Kumagai 2002; Kinlough et al. 2005). The side chain of a strictly conserved threonine (Thr391 in *E. coli* γ-GT, *Ec*GT) of the precursor protein acts as nucleophile for the cleavage, turning into the new N-terminal residue of the small subunit and then acting also as the catalytic nucleophile in the enzymatic reaction (Boanca et al. 2006; Lyu et al. 2009). Upon cleavage, the C-terminal segment of the large subunit (I378-Q390 in *Ec*GT) moves away from the threonine, thus forming the γ-glutamyl binding pocket. Meanwhile, the lid loop (residues 438–449 of *Ec*GT) forms a lid upon the pocket. When the pocket is occupied by a substrate or inhibitor the lid loop shields the catalytic pocket from the solvent, otherwise, when the pocket is empty the lid loop is disordered (Okada et al. 2006, 2007; Williams et al. 2009).

Recently, bacterial γ-GTs have been considered useful tools to produce γ-glutamyl compounds, more soluble and stable with respect to the non-modified precursors (Suzuki et al. 2007) or to be employed as glutaminase in food industry (Vermeulen et al. 2007). Although several γ-GTs from bacteria have been studied (Okada et al. 2006; Lin et al. 2006; Wada et al. 2010), little is known about extremophilic members of this family. Extremophiles are microorganisms adapted to live in hostile environments characterized by extreme pH, temperature, pressure or salt conditions and represent useful sources of stable enzymes for biotechnological applications. Extremophilic bacterial γ-GTs represent models to study the structure/function relationships of proteins adapted to extreme environments, as well as they can display useful properties for biotechnological applications.

We have previously reported the expression, purification and biochemical characterization of the γ-GT from the thermophilic bacterium *Geobacillus thermodenitrificans* (*Gth*GT). This protein is more stable than other bacterial γ-GTs and displays γ-glutamyl hydrolase activity, but it does not exhibit transpeptidase activity (Castellano et al. 2010). Recently, the X-ray structures of the γ-GTs identified from the genome sequences of other two extremophilic microorganisms, the thermoacidophilic archaeon *Thermoplasma acidophilum* (Ruepp et al. 2000) and the alkaliphilic bacterium *Bacillus halodurans* (Takami et al. 2000) have been only deposited in Protein Data Bank (pdb code 2I3O and 2NLZ), but not characterized. Interestingly, a common feature of all these extremophilic γ-GTs is the absence of residues corresponding to the lid loop.

Here, we report the purification and analysis of some molecular and structural properties of other two γ-GTs from extremophilic sources, i.e. the γ-GT from *Thermus thermophilus* (*Tt*GT) and that from *Deinococcus radiodurans* (*Dr*GT). *T. thermophilus* is a Gram-negative, aerobic eubacterium growing in the 50–82°C temperature range (Henne et al. 2004). *D. radiodurans* is one of the most radio-resistant microorganisms and it is known as a polyextremophile, as it tolerates cold, dehydration, vacuum, and acid (White et al. 1999).

*Tt*GT and *Dr*GT are both synthesized as precursor proteins that undergo an intramolecular autocatalytic cleavage to yield a large and a small subunit, respectively. Like *Gth*GT, these two extremophilic enzymes display γ-glutamyl hydrolase but not transpeptidase activity, using the standard acceptor substrate glycyl-glycine. The comparison between sequence and structural properties of the extremophilic γ-GTs and of their mesophilic counterparts shows that some residues, known to be involved in the catalytic action, are equally substituted in the examined extremophilic γ-GTs, thus suggesting that these proteins have used a common strategy to adapt to different hostile environments. Moreover, a phylogenetic analysis based on known γ-GTs sequences of different organisms from bacteria to mammals suggests that extant γ-GTs are evolved from enzymes that lack the residues corresponding to the lid loop. The possibility that γ-GTs are evolved from enzymes specialized in γ-glutamyl hydrolase activity is also discussed.

Materials and methods

Materials

L-Glutamic acid γ-(4-nitroanilide), glycyl-glycine, bovine serum albumin, kanamycin and γ-glutamyltranspeptidase

Type IV from Equine Kidney were from Sigma. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was from Inalco. Restriction enzymes were from New England Biolabs. PfuTurbo DNA polymerase and dNTP mix were from Promega. All other reagents were of analytical grade.

Construction of plasmids

The strains of *Thermus thermophilus* HB27 and *Deinococcus radiodurans* R1 were provided by DSMZ. The genomic DNA of the two bacterial strains was prepared using the GeneElute Bacterial Genomic DNA Kit from SIGMA. The complete coding sequences of the *Tt*GT and *Dr*GT genes (TTC0403 and DR1524) were amplified by PCR using the genomic DNA of the corresponding microorganism as template. For amplification of *Tt*GT the forward primer was 5′d-AGGGGGATGCATATGGACCT CACCTACTAC-3′ and the reverse primer was 5′d-T TCGCCTTCAAAGCTTCAAACTACCACGCC-3′. The *Nde*I and *Hin*dIII sites are underlined in the forward and reverse primer, respectively. For amplification of *Dr*GT the forward primer was 5′d-GGAATTCCATATGCTGCGG CCCATGACCCA-3′ and the reverse primer was 5′d-C CGCTCGAGTCACCAGAGCGCGATGTGTC-3′. The *Nde*I and *Xho*I sites are underlined in the forward and reverse primer, respectively. The PCR products were first cloned into the pGEM T-easy plasmid (Promega) and sequenced by PRIMM to confirm the identity of the two genes. After digestion with the restriction endonucleases, the fragments corresponding to the coding genes were ligated in frame into the similarly digested pET-28b expression vector (Novagen), in order to create two recombinant proteins, containing an N-terminal histidine tag.

Expression and purification of recombinant proteins

For the production of the recombinant proteins, *E. coli* BL21(DE3) cells, harboring the expression plasmids, were cultured in Luria–Bertani (LB) medium, containing 30 mg/L kanamycin and allowed to grow at 37°C until the OD_{600} reached the value of 0.6. Protein expression was induced by adding IPTG to a final concentration of 0.2 mM. Bacterial cultures were allowed to grow up to 4 h, at 22°C for the production of *Dr*GT and at 30°C for the production of *Tt*GT. Cells were then collected by centrifugation (5,000 rpm, 20 min, 4°C) and the pellet was lysed in 20 mM Tris–HCl, pH 8.0 by sonication. The cell homogenate was centrifuged at 30,000×*g* for 1 h and the supernatant was used as starting material for the purification of the soluble recombinant proteins by affinity chromatography, using the HIS-Nickel Affinity Gel (SIGMA). To improve the purification of *Dr*GT and *Tt*GT, cell extracts were treated at 45°C for 15 min prior to loading on the affinity chromatography. The fractions containing *Dr*GT or *Tt*GT were eluted with 20 mM Tris–HCl pH 8.0, 50 mM Imidazole. The cleavage of the N-terminal hexahistidine tag of the two recombinant proteins by incubation with thrombin did not alter the enzymatic activity. Therefore, further experiments were carried out on samples not treated with the protease. The purified proteins were concentrated by centricon (Amicon), dialyzed against 20 mM Tris–HCl, pH 8.0, and stored at 4°C. Protein concentration was determined using the BioRad Protein assay, based on the Bradford method (Bradford 1976), using bovine serum albumin as standard.

SDS-PAGE and N-terminal analysis of recombinant proteins

The expression of the recombinant proteins was examined by SDS-PAGE carried out according to Laemmli (1970). Samples were dissolved in buffer containing 5% β-mercaptoethanol. Gels were stained with Coomassie Blue R-250. The proteins separated on SDS-PAGE 12% were electroblotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was stained with 0.025% Coomassie Blue R-250 in 40% methanol, followed by destaining with 50% methanol. The protein bands were cut out and then applied to an automated protein sequencer to determine N-terminal sequence by automated Edman degradation.

Enzymatic activity determination

The enzymatic hydrolase activity was measured using the substrate analog L-glutamic acid γ-(4-nitroanilide) (GNA) (Tate and Meister 1985). The release of 4-nitroaniline was monitored at 412 nm and concentrations were determined using the reported extinction coefficient of 8,800 M^{-1} cm^{-1}. As the activity of the recombinant proteins was too low to allow continuous monitoring, the release of 4-nitroaniline was assessed by end point assay after 10 min of incubation with the substrate. For standard assays, hydrolysis activity was measured in 0.1 M Tris–HCl, pH 8.0, containing 1 mM GNA at 37 and 50°C using a Cary 50 spectrophotometer. Prior to proceeding with the enzymatic assay, *Tt*GT sample was incubated at 37°C overnight to obtain a full processing of the enzyme. All the determinations were done in triplicate on two different preparations of both enzymes. The extent of the transfer reaction was estimated from ratio between reactions with or without the standard acceptor glycyl-glycine. For the transpeptidation reaction 20 mM of glycyl-glycine was added to the assay mixture. 1 Unit was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per

min. γ-GT from equine kidney was used as positive control. For evaluating protein thermal stability, *Dr*GT and *Tt*GT were incubated at 50°C in 0.1 M Tris–HCl, pH 8.0 buffer; aliquots of the reaction were removed at appropriate times and incubated on ice for 10 min before measuring the residual activity at 50°C using 1 mM GNA as substrate.

Homology modeling

*Dr*GT and *Tt*GT monomers were obtained by homology modeling using the SWISS-MODEL server (http://swissmodel.expasy.org/) using the structure of the putative γ-GT from *Bacillus halodurans* (*Bh*GT, PDB code 2NLZ) as the template. The *Z*-score values of the combined statistical potential energy of the two models were −10.65 and −11.62, analogous of that of the template which is equal to −11.60. To further validate the models, the PSI-PRED server (Bryson et al. 2005) was used to perform predictions on type and position of secondary structure elements on *Dr*GT and *Tt*GT that were compared with those present on the models. The mature forms of *Dr*GT and *Tt*GT are assumed to be similar to that of *Gth*GT, *Ec*GT and *Hp*GT. The models of the mature forms of these two enzymes were thus obtained using the procedure already described for the building of the *Gth*GT model (Castellano et al. 2010). Briefly, the procedure is based on a manual superimposition of the large and the small subunit of each monomer on the respective parts of the template using the program O (Jones et al. 1990). The final models were then energy-minimized in vacuo by means of the GROMOS96 forcefield (van der Spoel et al. 1994) to relieve steric clashes. The structures were validated with PROSA web server (Wiederstein and Sippl 2007). The stereochemical quality of the final models showed that only three and five residues were positioned in the disallowed regions of the Ramachandran plot for *Dr*GT and *Tt*GT, respectively.

Computational studies of accessible surface, total surface and volumes were performed using VADAR web server (Willard et al. 2003). The total number of hydrogen bonds was determined with HBPLUS (McDonald and Thornton 1994). Cartoons were generated using Pymol (http://www.pymol.org).

Phylogenetic analysis

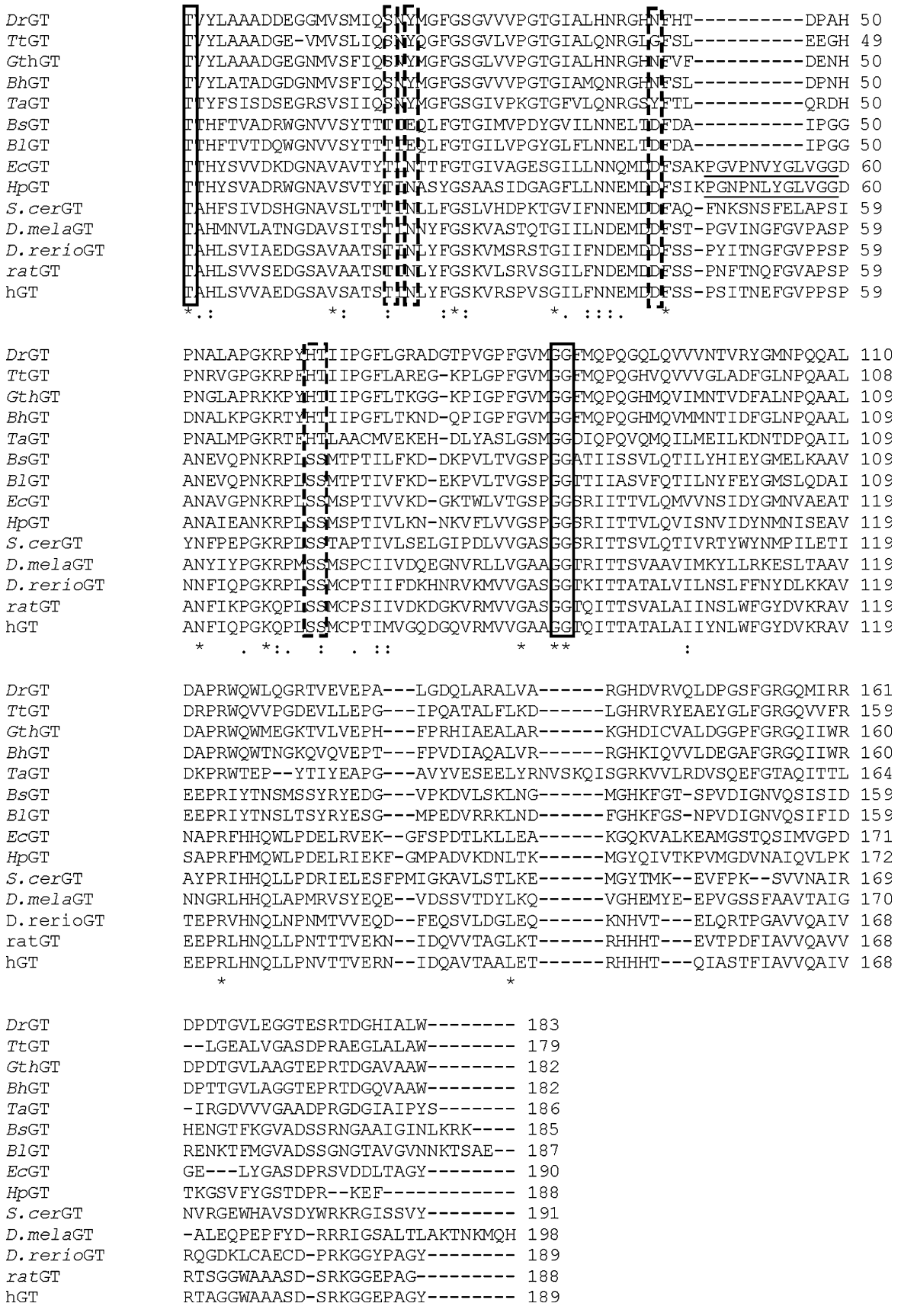
The distance-based tree reconstruction was performed with the Neighbor-Joining (NJ) method with the alignment generated by ClustalX 1.8, using the BLOSUM weight matrix. Positions with gaps were excluded from the alignment. The observed distances were corrected for multiple substitutions using Matoo Kimura's formula. 1,000 bootstrap replicates were made. The reliable branch nodes are supported by bootstrap values higher than 50%.

Fig. 1 Multiple sequence alignment of *Tt*GT and *Dr*GT with other bacterial and eukaryal homologues. The amino acid sequences of the small subunits of *Tt*GT and *Dr*GT were aligned with those of *Gth*GT (YP001127364.1), *B. halodurans* (NP241733.1), *T. acidophilum* (NP394454.1), *E. coli* (P18956), *H. pylori* (O25743), *B. subtilis* (P54422), *B. licheniformis* (A9YTT0), *D. rerio* (Q7T2A1), *D. melanogaster* (Q9VWT3), *S. cerevisiae* (Q05902) *R. norvegicus* (P07314) and *H. sapiens* (P19440) by the ClustalW method (Larkin et al. 2007). The conserved threonine residues (T346 and T353 in *Tt*GT and *Dr*GT, respectively) responsible for autoprocessing of the enzyme and the two strictly conserved glycine residues involved in binding of the γ-glutamyl moiety (G483 and G484 in *Ec*GT) are *boxed* in *continuous line*. The other residues responsible for substrate binding and catalytic activity of γ-GTs, but not conserved in extremophilic γ-GTs, are *boxed* in *dotted line* (T409, Y411, D433, S462 and S463 in *Ec*GT). The lid loop extending towards the active site (spanning from P438 to G449 of *Ec*GT) and absent in extremophilic γ-GTs and *Bs*GT is *underlined*

Results and discussion

Sequence analyses of extremophilic γ-GTs and comparison with bacterial and eukaryotic homologues

From the available genome sequences of *T. thermophilus* HB27 and *D. radiodurans* R1 (Henne et al. 2004; White et al. 1999), the open reading frames corresponding to the gene of 1,575 bp coding for the putative *Tt*GT and that corresponding to the gene of 1,608 bp coding for the putative *Dr*GT were identified. The two proteins consist of 525 and 536 amino acids, with a calculated molecular mass of 56,680 and 56,922 Da, respectively. The amino acid sequences show >50% identity with γ-GTs from extremophilic bacteria such as *Gth*GT and the putative γ-GT from *B. halodurans* (*Bh*GT); a lower sequence identity (>25%) was found with the bacterial γ-GTs from *E. coli* (*Ec*GT), *B. subtilis* (*Bs*GT), *H. pylori* (*Hp*GT), the archaeal γ-GT from *T. acidophilum* (*Ta*GT) and the eukaryotic γ-GTs, including human γ-GT (*h*GT). The multiple sequence alignment of γ-GTs shows that *Tt*GT and *Dr*GT have an overall primary structure similar to that of the well-described mammalian and bacterial γ-GTs (Fig. 1). The putative internal cleavage sites of *Tt*GT and *Dr*GT, leading to a large and a small subunit, are the strictly conserved Thr346 and Thr353, respectively. The sequence of the small subunit has previously been shown to contain the catalytic site of the enzyme and is slightly more conserved than that of the large subunit. The alignment of the small subunits of the above-mentioned proteins emphasizes the presence of highly conserved and potentially reactive amino acids (Fig. 1). Besides the above-mentioned strictly


DrGT TVYLAAADDEGGMVSMIQSNYMGFGSGVVVPGTGIALHNRGHNFHT---------- DPAH 50
TtGT TVYLAAADGE-VMVSLIQSNYQGFGSGVLVPGTGIALQNRGLGFSL---------- EEGH 49
GthGT TVYLAAADGEGNMVSFIQSNYMGFGSGLVVPGTGIALHNRGHNFVF---------- DENH 50
BhGT TVYLATADGDGNMVSFIQSNYMGFGSGVVVPGTGIAMQNRGHNFSL---------- DPNH 50
TaGT TTYFSISDSEGRSVSIIQSNYMGFGSGIVPKGTGFVLQNRGSYFTL---------- QRDH 50
BsGT TTHFTVADRWGNVVSYTTTIEQLFGTGIMVPDYGVILNNELTDFDA---------- IPGG 50
BlGT TTHFTVTDQWGNVVSYTTTIEQLFGTGILVPGYGLFLNNELTDFDA---------- IPGG 50
EcGT TTHYSVVDKDGNAVAVTYTNTTFGTGIVAGESGILLNNQMDDFSAKPGVPNVYGLVGGD 60
HpGT TTHYSVADRWGNAVSVTYTNASYGSAASIDGAGFLLNNEMDDFSIKPGNPNLYGLVGGD 60
S.cerGT TAHFSIVDSHGNAVSLTTTLNLLFGSLVHDPKTGVIFNNEMDDFAQ-FNKSNSFELAPSI 59
D.melaGT TAHMNVLATNGDAVSITSTLNNYFGSKVASTQTGIILNDEMDDFST-PGVINGFGVPASP 59
D.rerioGT TAHLSVIAEDGSAVAATSTLNLYFGSKVMSRSTGIIFNDEMDDFSS-PYITNGFGVPPSP 59
ratGT TAHLSVVSEDGSAVAATSTLNLYFGSKVLSRVSGILFNDEMDDFSS-PNFTNQFGVAPSP 59
hGT TAHLSVVAEDGSAVSATSTLNLYFGSKVRSPVSGILFNNEMDDFSS-PSITNEFGVPPSP 59
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DrGT PNALAPGKRPYHTIIPGFLGRADGTPVGPFGVMGGFMQPQGQLQVVVNTVRYGMNPQQAL 110
TtGT PNRVGPGKRPFHTIIPGFLAREG-KPLGPFGVMGGFMQPQGHVQVVVGLADFGLNPQAAL 108
GthGT PNGLAPRKKPYHTIIPGFLTKGG-KPIGPFGVMGGFMQPQGHMQVIMNTVDFALNPQAAL 109
BhGT DNALKPGKRTYHTIIPGFLTKND-QPIGPFGVMGGFMQPQGHMQVMMNTIDFGLNPQAAL 109
TaGT PNALMPGKRTFHTLAACMVEKEH-DLYASLGSMGGDIQPQVQMQILMEILKDNTDPQAIL 109
BsGT ANEVQPNKRPLSSMTPTILFKD-DKPVLTVGSPGGATIISSVLQTILYHIEYGMELKAAV 109
BlGT ANEVQPNKRPLSSMTPTIVFKD-EKPVLTVGSPGGTTIIASVFQTILNYFEYGMSLQDAI 109
EcGT ANAVGPNKRPLSSMSPTIVVKD-GKTWLVTGSPGGSRIITTVLQMVVNSIDYGMNVAEAT 119
HpGT ANAIEANKRPLSSMSPTIVLKN-NKVFLVVGSPGGSRIITTVLQVISNVIDYNMNISEAV 119
S.cerGT YNFPEPGKRPLSSTAPTIVLSELGIPDLVVGASGGSRITTSVLQTIVRTYWYNMPILETI 119
D.melaGT ANYIYPGKRPMSSMSPCIIVDQEGNVRLLVGAAGGTRITTSVAAVIMKYLLRKESLTAAV 119
D.rerioGT NNFIQPGKRPLSSMCPTIIFDKHNRVKMVVGASGGTKITTATALVILNSLFFNYDLKKAV 119
ratGT ANFIKPGKQPLSSMCPSIIVDKDGKVRMVVGASGGTQITTSVALAIINSLWFGYDVKRAV 119
hGT ANFIQPGKQPLSSMCPTIMVGQDGQVRMVVGAAGGTQITTATALAIIYNLWFGYDVKRAV 119
* . *:. : . :: * ** :
DrGT DAPRWQWLQGRTVEVEPA---LGDQLARALVA------RGHDVRVQLDPGSFGRGQMIRR 161
TtGT DRPRWQVVPGDEVLLEPG---IPQATALFLKD------LGHRVRYEAEYGLFGRGQVVFR 159
GthGT DAPRWQWMEGKTVLVEPH---FPRHIAEALAR------KGHDICVALDGGPFGRGQIIWR 160
BhGT DAPRWQWTNGKQVQVEPT---FPVDIAQALVR------RGHKIQVVLDEGAFGRGQIIWR 160
TaGT DKPRWTEP--YTIYEAPG---AVYVESEELYRNVSKQISGRKVVLRDVSQEFGTAQITTL 164
BsGT EEPRIYTNSMSSYRYEDG---VPKDVLSKLNG------MGHKFGT-SPVDIGNVQSISID 159
BlGT EEPRIYTNSLTSYRYESG---MPEDVRRKLND------FGHKFGS-NPVDIGNVQSIFID 159
EcGT NAPRFHHQWLPDELRVEK--GFSPDTLKLLEA------KGQKVALKEAMGSTQSIMVGPD 171
HpGT SAPRFHMQWLPDELRIEKF-GMPADVKDNLTK------MGYQIVTKPVMGDVNAIQVLPK 172
S.cerGT AYPRIHHQLLPDRIELESFPMIGKAVLSTLKE------MGYTMK--EVFPK--SVVNAIR 169
D.melaGT NNGRLHHQLAPMRVSYEQE--VDSSVTDYLKQ------VGHEMYE-EPVGSSFAAVTAIG 170
D.rerioGT TEPRVHNQLNPNMTVVEQD--FEQSVLDGLEQ------KNHVT---ELQRTPGAVVQAIV 168
ratGT EEPRLHNQLLPNTTTVEKN--IDQVVTAGLKT------RHHHT---EVTPDFIAVVQAVV 168
hGT EEPRLHNQLLPNVTTVERN--IDQAVTAALET------RHHHT---QIASTFIAVVQAIV 168
* *
DrGT DPDTGVLEGGTESRTDGHIALW-------- 183
TtGT --LGEALVGASDPRAEGLALAW-------- 179
GthGT DPDTGVLAAGTEPRTDGAVAAW-------- 182
BhGT DPTTGVLAGGTEPRTDGQVAAW-------- 182
TaGT -IRGDVVVGAADPRGDGIAIPYS------- 186
BsGT HENGTFKGVADSSRNGAAIGINLKRK---- 185
BlGT RENKTFMGVADSSGNGTAVGVNNKTSAE-- 187
EcGT GE---LYGASDPRSVDDLTAGY-------- 190
HpGT TKGSVFYGSTDPR--KEF------------ 188
S.cerGT NVRGEWHAVSDYWRKRGISSVY-------- 191
D.melaGT -ALEQPEPFYD-RRRIGSALTLAKTNKMQH 198
D.rerioGT RQGDKLCAECD-PRKGGYPAGY-------- 189
ratGT RTSGGWAAASD-SRKGGEPAG--------- 188
hGT RTAGGWAAASD-SRKGGEPAGY-------- 189

conserved threonine residues, the two Gly residues (Gly427-Gly428 in *Tt*GT and Gly436-Gly437 in *Dr*GT), already proposed to have a role in the stabilization of the tetrahedral transition state of the enzyme (Boanca et al. 2007), are perfectly conserved. On the other hand, other residues, considered to be important for the catalysis in *Ec*GT and *Hp*GT, are equally substituted in *Tt*GT and *Dr*GT, as well as in *Gth*GT. In particular, Thr398 (numbering scheme of *Hp*GT), reported to enhance the nucleophilicity of the catalytic threonine (Boanca et al. 2007), is substituted by a Ser (Ser371 in *Dr*GT and Ser363 in *Tt*GT), whereas Ser462 and Ser463 (numbering scheme of *Ec*GT) are replaced by a His and a Thr in the two extremophilic γ-GTs. Furthermore, Asp433 and Arg114 (numbering scheme of *Ec*GT), whose mutations have been reported to drastically reduce the transpeptidase activity of the enzyme (Minami et al. 2003), are replaced by Gly387 and Gln395, Ser83 and Ser87 in *Tt*GT and *Dr*GT, respectively. Another important difference of *Tt*GT and *Dr*GT, as well as of *Gth*GT, when compared with other γ-GTs, is the absence of the lid loop extending towards the active site. This area, well conserved in all eukaryotic γ-GTs and in several bacterial counterparts, should play a role in regulating the access of the substrate to the active site or the binding of the substrate to the active site cleft. The absence of the lid loop is also a characteristic of *Bs*GT and of the putative *Bh*GT and *Ta*GT. This feature has been associated with an active site cleft more exposed to the solvent with respect to that observed in the other known γ-GTs (Wada et al. 2010). Finally, the C terminus, which in *Hp*GT has been demonstrated to contribute to optimal enzymatic efficiency and autoprocessing of the enzyme, shows a low sequence conservation (Williams et al. 2009).

Protein expression and purification

Two prokaryotic expression vectors containing the coding sequences of the *Tt*GT and *Dr*GT genes were generated and inserted in *E. coli* BL21(DE3) cells for expression. The soluble portion of the recombinant proteins was purified by Ni-affinity chromatography exploiting the engineered N-terminal hexahistidine tag. The molecular properties of the recombinant proteins were analyzed by SDS/PAGE under reducing conditions. *Dr*GT was purified as a fully mature enzyme, displaying two major bands of 40 and 20 kDa, respectively. Otherwise, *Tt*GT was purified as a mixture of the unprocessed and mature enzyme, exhibiting three major bands of 59, 40 and 19 kDa, respectively (Fig. 2). The theoretical molecular masses for large and small subunits are 37.352 and 19.588 kDa for *Dr*GT, 37.701 18.996 kDa for *Tt*GT. However, the precursor and the large subunit exhibited a higher apparent molecular weight due to the presence of the N-terminal histidine tag. To verify that

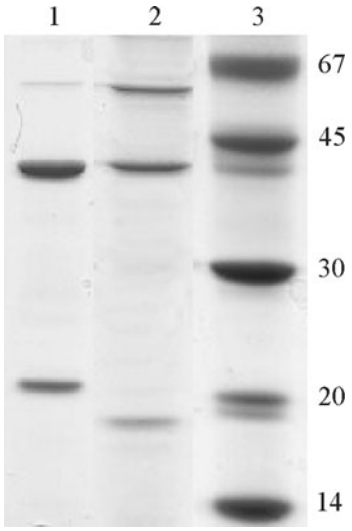

Fig. 2 Coomassie stained gel after partial purification of recombinant *Dr*GT and *Tt*GT. Samples were run on a 12% SDS-PAGE gel under reducing conditions. *1*: 3 μg of purified recombinant *Dr*GT. *2*: 3 μg of purified recombinant *Tt*GT. *3*: Molecular weight standards (Amersham Biosciences) in kDa

*Dr*GT is synthesized as a precursor form of 60 kDa undergoing autoprocessing to yield a large (40 kDa) and a small (20 kDa) subunit, the amino-terminal sequence of the two peptides was determined by Edman degradation of the electroblotted recombinant protein. The N-terminal sequence of the 40 kDa peptide from *Dr*GT was MLRPM, whereas the amino-terminal sequence of the 20 kDa peptide was TVYLA. The resulting N-terminal residue (Thr353) of the 20 kDa subunit was confirmed to be the threonine residue perfectly conserved in the other γ-GTs (Fig. 1).

Biochemical properties of *Tt*GT and *Dr*GT

The recombinant proteins *Tt*GT and *Dr*GT were first studied for the γ-glutamyl amide bond hydrolase activity. The hydrolysis reaction was measured using 1 mM GNA as the donor substrate, in the absence of an acceptor. The specific activities of *Tt*GT and *Dr*GT, measured at 37 and 50°C, pH 8, were compared with those of *Gth*GT and reported in Table 1. γ-GT from equine kidney was used as positive control. The hydrolase activity of the three extremophilic enzymes were found to be lower than that of the mammalian counterpart, as expected for the substitution in these proteins of some amino acid residues essential for enzyme catalysis (Ikeda et al. 1995). Moreover, the hydrolase activity was found to be increased at 50°C, as expected for thermophilic proteins. Unfortunately, the instability of the GNA substrate prevented a good measurement of the specific activity at higher temperatures.

*Tt*GT and *Dr*GT were also studied for their transpeptidase activity. The ratio between transpeptidase and

Table 1 Specific hydrolase and transpeptidase activities of γ-GTs from extremophilic enzymes

Enzyme	Hydrolase activity (U/mg) at 37°C	Transpeptidase activity (U/mg) at 37°C	Transpeptidation/ hydrolysis at 37°C	Hydrolase activity (U/mg) at 50°C	Transpeptidase activity (U/mg) at 50°C	Transpeptidation/ hydrolysis at 50°C
*Tt*GT	0.129	<0.001	<0.001	0.151	<0.001	<0.001
*Dr*GT	0.047	0.005	0.106	0.060	0.006	0.1
*Gth*GT	0.113[a]	0.002[a]	0.018[a]	0.245[a]	0.009[a]	0.037[a]
*Eq*GT[b]	2.318	11.460	4.944	3.728	6.403	1.718

[a] Data previously obtained from biochemical characterization of *Gth*GT (Castellano et al. 2010)

[b] *Eq*GT, γ-glutamyltranspeptidase Type IV from Equine Kidney used as positive control

hydrolase activity of *Dr*GT was close to zero, whereas that of *Tt*GT was undetectable (Table 1). Therefore, the two extremophilic enzymes did not exhibit transpeptidase activity at the indicated conditions even at high concentrations of the acceptor. In the same conditions the γ-GT from equine kidney displayed a transpeptidase activity fivefold increased at 37°C with respect to its hydrolase activity. These data indicate that *Tt*GT and *Dr*GT do not function as general transpeptidases, even though a broad range of further acceptor substrates should be investigated. Since the absence of transpeptidase function has been also observed in *Gth*GT (Castellano et al. 2010), it can be speculated that the inability to catalyze the transfer of glutamate to the acceptor amino acids or peptides may be a feature of many other extremophilic γ-GTs. In this respect, it should be mentioned that significant catalytic differences have been already reported between bacterial and eukaryotic homologues; for example, *Hp*GT was found to be 100-fold less effective at catalyzing transpeptidation with respect to human γ-GT (Boanca et al. 2006).

Finally, we evaluated the residual hydrolase activity of *Tt*GT and *Dr*GT after 20 h incubation at 50°C and found that the two enzymes retain 53 and 83% of the initial activity, respectively. These data confirm that also these two extremophilic γ-GTs, as well as *Gth*GT (Castellano et al. 2010), are more thermostable than their mesophilic counterparts.

Homology modeling of *Dr*GT and *Tt*GT

The structures of the mature forms of *Dr*GT and *Tt*GT were modeled with a protocol similar to that adopted for *Gth*GT (Castellano et al. 2010), using as template the available 3D crystallographic structure of the putative *Bh*GT. The structure of the putative *Bh*GT has been deposited in PDB, but has not been described in the literature. Positional sequence identity of *Dr*GT and *Tt*GT with *Bh*GT is 58 and 53%, respectively. The *Z*-score values of the combined statistical potential energy of the monomeric models used to build the mature form are −10.65 and −11.62, respectively. These values are in the range of scores typically found in proteins of similar sequence length and are analogous to the value of the template equal to −11.60. Similar to *Gth*GT, the predicted structures of *Dr*GT and *Tt*GT are characterized by a tertiary fold consisting of a stacked αββα structure with two antiparallel β-sheets between α-helical layers (Fig. 3a, b). This topology is similar to that observed in the other members of the family.

The Cα atoms of the mature form of *Dr*GT and *Tt*GT were superimposed to those of *Ec*GT and *Hp*GT with a root mean square (rms) deviation in the range 1.24–1.94 Å. This finding indicates that no major differences are present between *Dr*GT, *Tt*GT and the other γ-GTs in terms of global architecture. The main structural variations between the models of the two new extremophilic γ-GTs and those of the other known γ-GTs are located in the large subunits. In particular, in *Dr*GT, a loop (residues 97–99) replaces the short helix formed by residues 98–102 of *Bh*GT, whereas the helix formed by residues 156–173 of *Bh*GT appears significantly shorter (residues 152–161 of *Dr*GT). The latter is also shorter in the *Tt*GT model.

A common feature of the *Dr*GT and *Tt*GT structures is the absence of the lid loop (Fig. 3c) as shown by the comparison with *Ec*GT, this latter possessing lid loop and transpeptidase activity (Fig. 3d). This feature, as in the case of *Gth*GT and *Bs*GT, is responsible for a more solvent-exposed active site pocket in these enzymes with respect to that of the other γ-GTs.

Sequence and structural features of the extremophilic γ-GTs

To shed light on the molecular features responsible for the adaptation to extreme environments in γ-GTs, some parameters related to amino acid composition (Table 2) and the structure (Table 3) of *Tt*GT and *Dr*GT were analyzed and compared in detail with those of *Gth*GT and of other γ-GTs from mesophilic bacteria such as *Ec*GT, *Hp*GT and *Bs*GT.

Analysis of amino acid composition shows that the extremophilic proteins are more similar to each other than to the other γ-GTs. All the extremophilic γ-GTs show

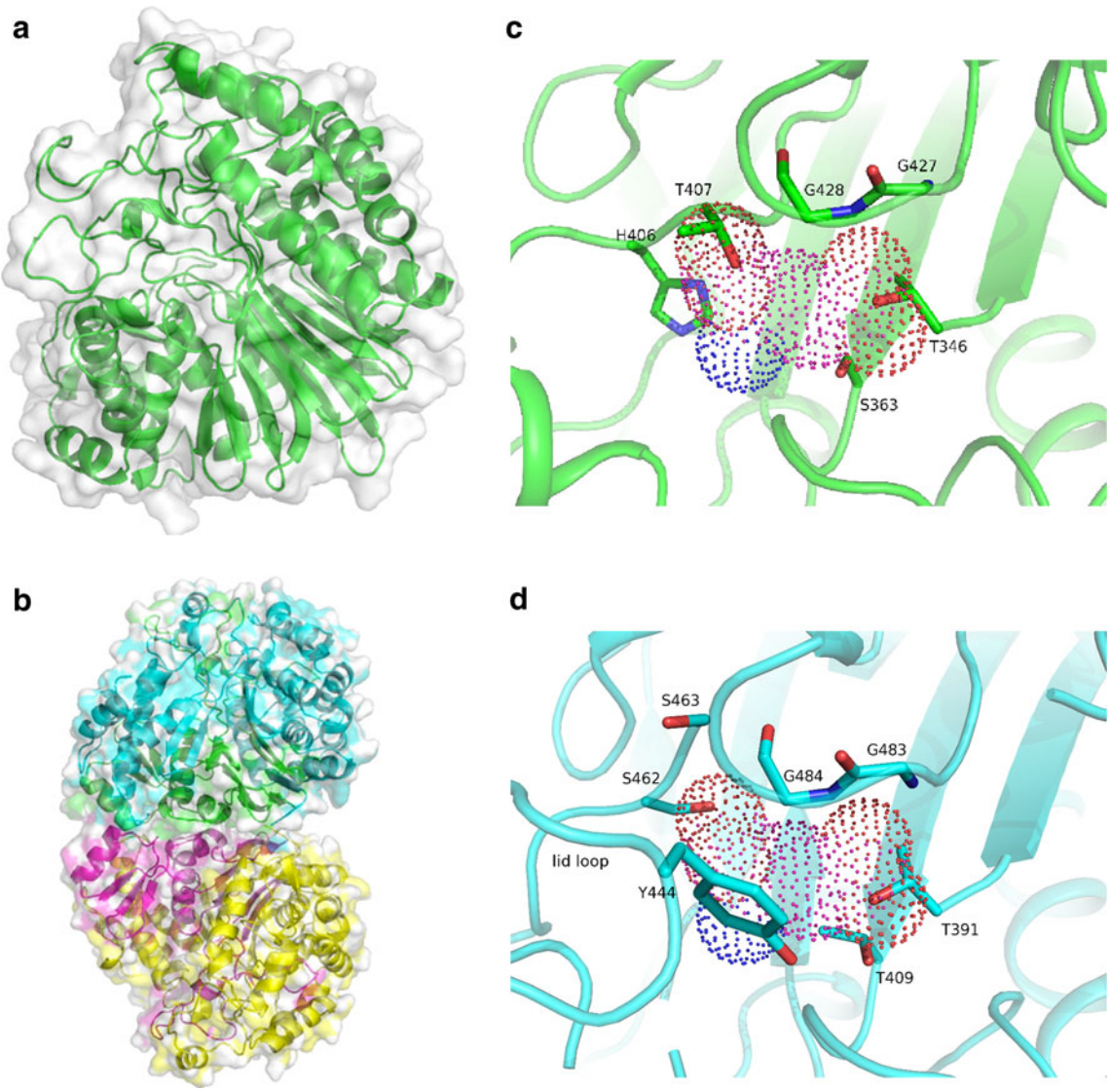

Fig. 3 Structure of monomeric (**a**) and mature form (**b**) of *Tt*GT. The large and small subunits are indicated in *cyan* and *yellow*, *green* and *purple*, respectively. The enlargements show the active site pockets of *Tt*GT in *green* (**c**) and *Ec*GT in *cyan* (**d**). The *dots* indicate the position of a substrate analog docked into the active site. For sake of clarity, some main chain atoms have been omitted (color figure online)

(Table 2) a decreased content of uncharged polar residues Gln, Asn, Thr and Ser with respect to *Ec*GT, *Hp*GT and *Bs*GT. Furthermore, the extremophilic γ-GTs show a significant enhancement of the Arg content and of the Arg/Lys ratio when compared to the other mesophilic γ-GTs. This feature has been previously linked to high temperatures adaptation (Szilagyi and Zavodsky 2000) since Arg residues have a higher hydrogen bonding potential than lysines, and the greater number of arginines enhances the thermal stability of the proteins. Among the examined extremophilic microorganisms, *D. radiodurans* grows at mesophilic temperature (30°C), but it has been considered "the world's toughest bacterium" as it can survive different stresses such as UV radiation, cold and acid. Therefore, we can speculate that these features reflect a general intrinsic stability of proteins adapted to resist to extreme conditions. In this respect, *Dr*GT shows the higher values for Arg content and Arg/Lys ratio and displays a higher thermal stability at 50°C with respect to *Tt*GT. Finally, it should be noted that extremophilic γ-GTs display a larger content of Gly and Pro residues when compared with mesophilic counterparts. These two residues usually occur in β-turn and the number of β-turns favors the compact state.

When comparing the structural features (Table 3), all the examined extremophilic γ-GTs show a decrease of total accessible surface area with respect to the mesophilic counterparts, coupled to lower values of exposed non-polar accessible surface area and fraction of non-polar accessible surface area and an increase of α-helix content and fraction

Table 2 Parameters related to the amino acid composition of mesophilic towards extremophilic γ-GTs

Large subunit	*Ec*GT	*Hp*GT	*Bs*GT	*Gth*GT	*Dr*GT	*Tt*GT
Number of protein residues	580	567	587	534	535	524
Molecular weight (Da)	61,785.9	61,151.3	64,111.8	57,668.5	56,922.2	56,680.0
Total number of uncharged polar residues Gln, Asn, Thr, Ser (%)	20.4	20.4	20.4	15.3	15.2	11.4
Total number of hydrophobic residues Ala + Val + Ile + Leu (%)	31.0	32.2	28.4	31.9	33.6	34.0
Number of Arg (R) (%)	2.8	2.8	2.7	4.1	7.1	8.0
Number of Lys (K) (%)	6.7	8.5	8.3	3.7	0.7	1.7
Arg/Lys	0.42	0.33	0.33	1.11	10.14	4.7
Arg/(Arg + Lys)	0.29	0.25	0.25	0.53	0.91	0.82
Total number of positively charged residues (Arg + Lys) (%)	9.5	11.3	11.0	7.8	7.8	9.7
Total number of negatively charged residues (Asp + Glu) (%)	11.4	9.1	13.0	10.1	9.4	10.9
(Asp + Glu)/(Arg + Lys)	1.20	0.81	1.18	1.29	1.20	1.12
Total number of charged residues Glu + Asp + Lys + Arg (%)	20.9	20.4	24.0	17.9	17.2	20.6
Total number of Gly (%)	9.7	9.5	9.2	10.7	12.5	12.2
Total number of Pro (%)	6.0	4.8	5.5	7.7	7.7	8.6

Table 3 Structural parameters of extremophilic γ-GTs towards mesophilic counterparts

Structural parameter	*Ec*GT	*Hp*GT	*Bs*GT	*Gth*GT	*Dr*GT	*Tt*GT
Total accessible surface area (ASA, Å^2)	38,250.0	37,709.3	37,176.4	36,829.4	35,010.0	36,798.1
Accessible surface area of backbone (Å^2)	5,068.4	4,535.4	5,353.2	4,463.5	4,411.9	4,452.8
Accessible surface area of side chains (Å^2)	33,181.6	33,174.0	31,823.3	32,365.9	30,598.1	32,345.3
Mean residue ASA (Å^2)	36.4	35.3	34.6	35.8	34.0	35.7
Exposed non-polar ASA (Å^2)	22,537.4	23,113.5	22,435.2	21,047.0	20,012.6	21,002.5
Exposed polar ASA (Å^2)	8,196.1	8,284.7	8,094.7	7,611.0	7,271.4	7,612.0
Exposed charged ASA (Å^2)	7,516.5	6,311.1	6,646.5	8,171.4	7,726.0	8,183.5
Fraction non-polar ASA (%)	59	61	60	57	57	57
Fraction polar ASA (%)	21	22	22	21	21	21
Fraction charged ASA (%)	20	17	18	22	22	22
Total volume (Å^3)	152,171.4	143,584.1	146,566.9	149,319.7	149,167.9	150,157.1
Mean residue volume (Å^3)	144.9	134.4	136.5	145.0	144.8	145.8
β-Sheet content (%)	24	23	25	24	25	25
α-Helix content (%)	33	36	36	37	37	37
Coil content (%)	43	41	39	39	38	38
Fraction residues with hydrogen bonds (%)	78	78	76	80	80	79

of residues with hydrogen bonds. All these features could be responsible for a higher compactness of extremophilic proteins to guard against different stress conditions. Moreover, extremophilic γ-GTs show higher values of exposed charged accessible surface area and fraction of charged accessible surface area than their mesophilic counterparts. Protein electrostatics interactions may play important roles for protein adaptation to different hostile environments. For example, extremophilic γ-GTs may use electrostatic interactions to preserve the assembly of the large and small subunits at high temperatures or to ensure proper solvation and avoid self-aggregation in acid conditions.

Phylogenetic analysis

To outline the evolutionary relationships of γ-GT protein family, a preliminary phylogenetic analysis was carried out from an alignment of the known sequences of γ-GT small subunits, which contain most of residues responsible for enzyme activity (Fig. 4). Among the extremophilic γ-GTs, also the archaeal *Ta*GT lacks the lid loop and the essential residues likely responsible for transpeptidase activity (see alignment in Fig. 1). Because of the lowest value of amino acid sequence identity shared with the other extremophilic γ-GTs, *Ta*GT was chosen as the outgroup.

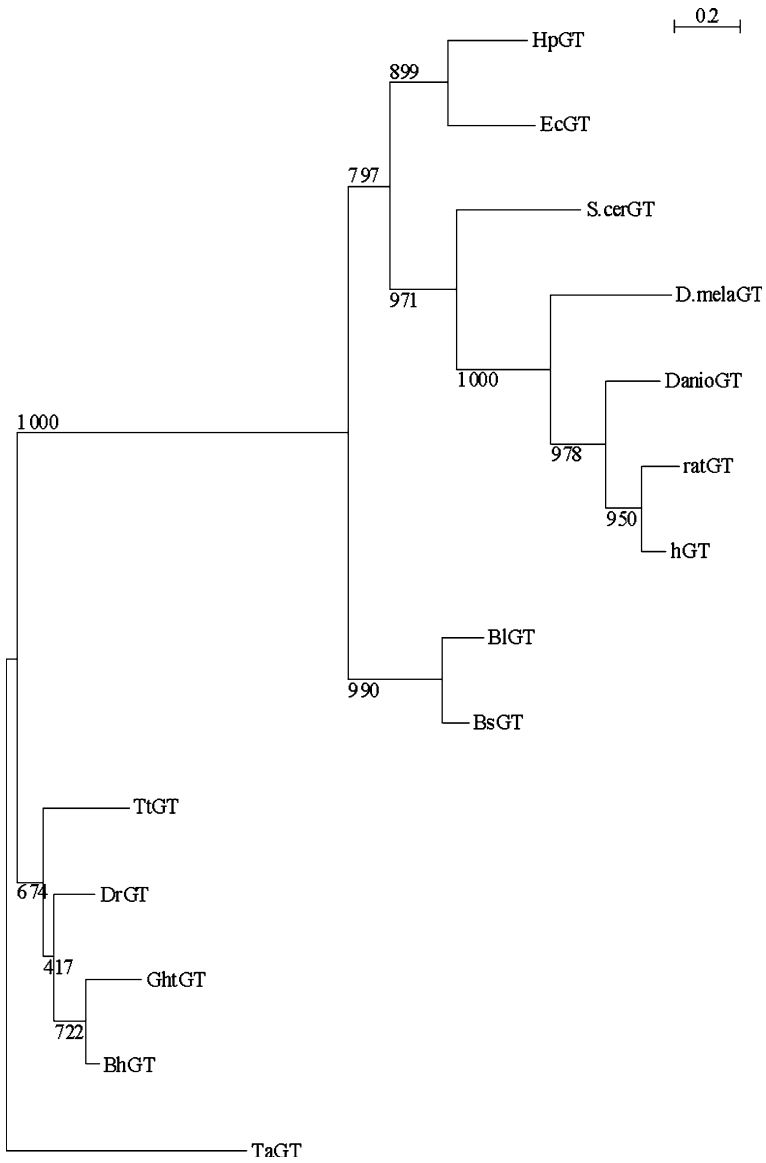

Fig. 4 Phylogram of γ-GTs. The amino acid sequences of γ-GTs small subunits used for the alignment were from *G. thermodenitrificans*, *T. thermophilus*, *D. radiodurans*, *B. halodurans*, *T. acidophilum*, *E. coli*, *H. pylori*, *B. subtilis*, *B. licheniformis*, *D. rerio*, *D. melanogaster*, *S. cerevisiae*, *R. norvegicus* and *H. sapiens*. Trees were produced by Clustal X (1.83.1) program using the Neighbor-Joining method and choosing the archaeal *Ta*GT as the outgroup

The phylogenetic tree suggests that γ-GTs from extremophilic sources evolved earlier and independently from the other bacterial and eukaryal counterparts, likely by specializing in hydrolase activity and adapting through several mutations to extreme environments. On the other hand, γ-GTs from non-extremophilic bacteria evolved later and developed also transpeptidase activity, then this activity reached the major effectiveness with eukarya. This hypothesis is in line with the findings that bacterial *Ec*GT and *Hp*GT are poor transpeptidases with respect to human γ-GT. It remains to be established whether the progenitor of these extant γ-GTs is endowed with lid loop or not. Interestingly, *Bs*GT and *Bl*GT lack the lid loop, but retain transpeptidase activity.

This analysis is restricted to a few numbers of characterized γ-GTs. Further studies on the activity of other members of this family may help to shed light on some unresolved questions: have the extant γ-GTs a common ancestor? Does this common ancestor lack the lid loop and/or transpeptidase activity? Did the presence of the lid loop and transpeptidase activity evolve independently?

Conclusions

In this study, two members of γ-GT family from two different extremophilic bacteria, *T. thermophilus* and *D. radiodurans*, were produced as recombinant proteins by an *E. coli* expression system, analyzed for enzymatic activity and some molecular and structural properties and compared with γ-GT from *G. thermodenitrificans* and mesophilic counterparts in order to find a common

'stabilization strategy' used by this enzyme family to adapt to extreme environments.

*Th*GT and *Dr*GT, as well as *Gth*GT, display γ-glutamyl hydrolase activity using GNA as a donor substrate, but they do not exhibit transpeptidase activity towards glycyl-glycine used as γ-glutamyl acceptor. Notably, the lack of transpeptidase activity in the reported conditions could represent an advantage for food industry, because these enzymes could function as glutaminase without generating γ-glutamyl compounds as by-products.

Structural and sequence analysis of *Tt*GT, *Dr*GT and comparison with *Gth*GT and other γ-GTs suggest that the presence of the strictly conserved Thr at the N terminus of the small subunit (Thr346 of *Tt*GT) and of the two Gly (residues 427 and 428 in *Tt*GT) at the oxyanion hole are essential, whereas substitution of other residues at the substrate binding site is tolerated, at least to maintain the hydrolase activity (Fig. 3c, d). Interestingly, some residues, reported to be involved in the enzyme function, are equally substituted in γ-GTs from extremophilic sources (Fig. 1). These substitutions could, in part, explain the lack of transpeptidase activity of these extremophilic enzymes and the reduced hydrolase activity with respect to other bacterial and mammalian counterparts. Furthermore, these γ-GTs lack the lid loop and this feature could allow the proteins to accommodate other substrates in the active site binding pocket.

One of the most important means of evolutionary adaptation to extreme conditions is the enhancement via mutations of the intrinsic stability of proteins (Szilagyi and Zavodsky 2000; De Vendittis et al. 2008). We suggest that the examined extremophilic γ-GTs have adopted similar molecular strategies to survive at hostile environments, including the increase of the charged residues on the protein surface. The determination of the X-ray structure of the extremophilic members of this family could expand our knowledge about these enzymes and confirm the stated hypotheses.

Finally, a phylogenetic tree, generated using a multiple sequence alignment of different γ-GTs from bacteria to eukarya suggests that extremophilic γ-GTs, lacking the lid loop and transpeptidase activity, could be the progenitors of the extant γ-GTs.

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