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REVIEW

Transglutaminases: part I—origins, sources, and biotechnological characteristics

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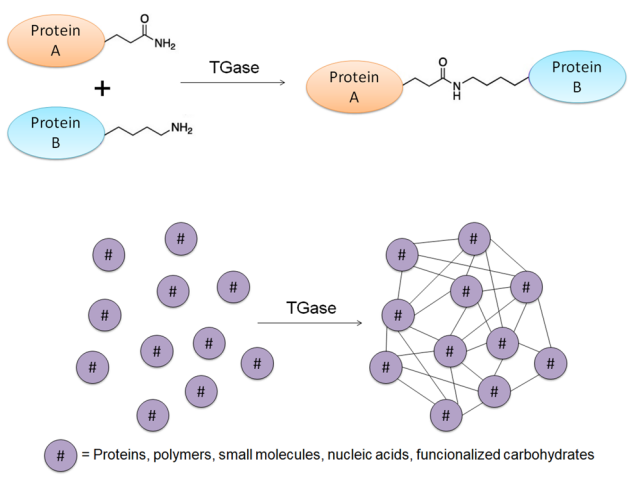
Abstract

The transglutaminases form a large family of intracellular and extracellular enzymes that catalyze cross-links between protein molecules. Transglutaminases crosslinking properties are widely applied to various industrial processes, to improve the firmness, viscosity, elasticity, and water-holding capacity of products in the food and pharmaceutical industries. However, the extremely high costs of obtaining transglutaminases from animal sources have prompted scientists to search for new sources of these enzymes. Therefore, research has been focused on producing transglutaminases by microorganisms, which may present wider scope of use, based on enzyme-specific characteristics. In this review, we present an overview of the literature addressing the origins, types, reactions, and general characterizations of this important enzyme family. A second review will deal with transglutaminases applications in the area of food industry, medicine, pharmaceuticals and biomaterials, as well as applications in the textile and leather industries.

Graphic abstract



Keywords Transglutaminase · Microbial transglutaminases · Protein cross-linking · *Bacillus subtilis* · *Streptomyces mobaraensis*

Introduction

Transglutaminase (EC 2.3.2.13, protein-glutamine gamma-glutamyltransferase, TGase) is a calcium-dependent enzyme, belonging to the class of transferases, which catalyzes the acyl-transfer between glutamine residues and a wide variety of primary amines (Ohtsuka et al. 2000). The reaction

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product results in stable, insoluble macromolecular complexes (Esposito and Caputo 2004). The formation of isopeptide bonds results in both intra- and inter-molecular cross-linking of proteins, the latter leading to protein polymerization (Griffin et al. 2002).

TGases are known to be widely distributed in nature, being found in vertebrates, invertebrates, mollusks, plants, and microorganisms (Folk 1980; Shleikin and Danilov 2011). They are involved in various physiological functions such as blood clotting, wound healing and epidermal keratinization, stabilization of photosynthetic complexes in the chloroplast, and programmed cell death in plants. Moreover, TGases are used extensively in the food industry, in tissue engineering, as well as in biochemical and biomedical research, and textile and leather processing (Griffin et al. 2002; Heck et al. 2013; Serafini-Fracassini and Del Duca 2008; Yang et al. 2011; Yokoyama et al. 2004; Zhu and Tramper 2008).

The term transglutaminase was first introduced in the literature by Clarke et al. (1959) when the authors found an enzyme showing transamidating properties in the guinea-pig liver (Clarke et al. 1959; Folk and Cole 1966). Until late 1980s, the TGase isolated from guinea-pig and from other mammalians blood were the most important sources of this enzyme (Clarke et al. 1957).

Mammalian TGases require Ca^{2+} for activation and show a red pigmentation, affecting the appearance of commercial products, thus none of these enzymes have ever been commercialized or accepted for industrial applications in food production, influencing the search for alternative, convenient commercial sources (Beninati et al. 2008; de Góes-Favoni and Bueno 2014; Jaros et al. 2006; Yokoyama et al. 2004).

In this context, the objective of part I of this review is to present a framework on transglutaminases of mammalian, non-mammalian (invertebrates, plants, fungi), and microbial origins, with special emphasis on the microbial enzymes because of their industrial importance. The properties of transglutaminases of *Streptoverticillium* and *Bacillus*—two of the most important sources of these enzymes—as well as the use of recombinant microorganisms for their production, are presented in detail. The uses and applications of transglutaminases in the food and biotechnology industries will be presented in a second part review on this subject.

Origins

The evolutionary history of TGase is not fully understood. However, given the similarities in the catalytic triad and the mechanism of transglutaminase reaction, is possible to suggests that transglutaminases have an evolutionary relationship to papain-like thiol proteases whose closest current representative is found in domain NlpC/P60. Clustering of the transglutaminase-like domains by sequence similarity identified a superfamily of proteins homologous to eukaryotic transglutaminases that are found in all archaea, some bacteria and yeast species, and the *Caenorhabditis elegans*. Sequence conservation involves the catalytic triad the transglutaminase. In 1999, Makarova et al. presented a computational analysis of this superfamily, remaining one of the most complete work comparing TGases among prokaryotes and eukaryotes. Since then, many more gene sequences of TGase have been published and a new phylogenetic tree based on the gene sequences are in preparation by our group and should be published soon (Anantharaman and Aravind 2003; Fernandes et al. 2015; Makarova et al. 1999).

In mammalian transglutaminases the catalytic mechanism is based on a triad of non-contiguous amino acids, i.e., Cys-His-Asp, and have the highly conserved active site region (GQCWVF) as can be seen in Fig. 1. Microbial transglutaminases show no similarity to mammalian transglutaminases, although they have the same catalytic triad, with a different sequence order, namely Cys-Asp-His (Giordano and Facchiano 2019; Kashiwagi et al. 2002; Whitaker et al. 2002).

Transglutaminase catalyzed reactions

The transamidation reactions catalyzed by TGase, including crosslinking, have attracted major research interests because of the potential applications in both the food and pharmaceutical industries. Amine incorporation and deamidation reactions are also well recognized because of their importance in transglutaminase-mediated post-translational modifications of proteins (Griffin et al. 2002; Lorand and Graham 2003; Yokoyama et al. 2004). The mechanism of action of TGase is the reversion of the proteolysis reaction catalyzed by the thiol proteases (Makarova et al. 1999; Plácido et al.

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GVSPMSWIGSVDILRRWKNHGCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQ   TG1
GRDPRSWNGSVEILKNWKKSGFSPVRYGQCWVFAGTLNTALRSLGIPSRVITNFNSAHDT   TG2
GTSPLHWRGSVAILQKWLKGRYKPVKYGQCWVFAGVLCTVLRCLGIATRVVSNFNSAHDT   TG3
GANPAEWTGSVAILKQWNATGCQPVRYGQCWVFAAVMCTVMRCLGIPTRVITNFDSGHDT   TG4
GVSPLEWKGSVAILQQWSARGGQPVKYGQCWVFASVMCTVMRCLGVPTRVVSNFRSAHNV   TG5
GTNPSAWVGSVEILLSYLRTG-YSVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDT   TG6
GVPPSAWTGSVDILLEYRSSE-NPVRYGQCWVFAGVFNTFLRCLGIPARIVTNYFSAHDN   TG7
GTAPYKWTGSAPILQQYYNTK-QAVCFGQCWVFAGILTTVLRALGIPARSVTGFDSAHDT  FXIIIa
GALLNKRRGSVPILRQWLTGRGRPVYDGQAWVLAAVACTVLRCLGIPARVVTTFASAQGT Band 4.2
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Fig. 1 Amino acid sequences near the active site of human TGases. Band 4.2 does not show TGase activity because it carries a Cys → Ala substitution at the active site

2008) and consists of two steps residue (Fig. 2a). In the first step the cysteine thiol group present in the active site of the enzyme attacks the side chain of the glutamine residue (acyl acceptor) on the protein substrate. In this way, the acyl-enzyme complex is formed with concomitant ammonia release. In the second step, reactions may occur in three different ways (Eckert et al. 2014; Gundemir et al. 2012; Lai et al. 2017; Lorand and Graham 2003; Yang et al. 2011):

I Crosslinking reaction between a γ-glutamyl containing peptide substrate and either a ε-amine group from a peptide-bound Lys residue (Fig. 2a, step 2, reaction I). This type of reaction is kinetically favored at pH > 7 and high substrate concentrations.
II When transglutaminase promotes the reaction between γ-glutamyl containing peptide substrate and the available primary amine substrate (such as biogenic amines), the enzyme catalyzes the incorporation of the primary amino group and resulting of a γ-glutamyl-amine bond (Fig. 2a, step 2, reaction II).
III When water acts as the acyl acceptor and the resultant hydrolysis reaction yields a glutamic acid (E) residue (Fig. 2a, step 2, reaction III).

All TGase may present high specificity in relation to glutamine substrates and low specify when compared to acyl-acceptor amino group. This acyl-acceptor amino group may have two types: the ε-amino group of the peptide lysine or the low-molecular primary amine (Shleikin and Danilov 2011). Figure 2b shows the possible catalytic mechanism for microbial transglutaminases (based on *S. mobaraensis*). The amino acid residues of mTGase active site are shown in step 1. In step 2, the thiolate ion of Cys producing a nucleophilic attack to an acyl donor, the side chain of the Gln residue (substrate 1). In steps 3 and 4, Asp donates a proton to the resultant oxyanion intermediate, and an ammonium is released. In step 5, an acyl acceptor, such as the side chain of the Lys residue (substrate 2), approaches the active site, and the side chain of Asp, which is now negatively charged, causing a nucleophilic attack to one proton of the acyl acceptor. Finally, in steps 6 and 7, the product is released from the resultant oxyanion intermediate, and the catalytic reaction is finished, and the catalytic site is released for a new reaction, returning to step 1 (Kashiwagi et al. 2002). In this mechanism, the Asp residue plays the role of His residue for Factor XIII transglutaminases (Pedersen et al. 1994).

Methods for measuring TGase activity

There are many assay methods for measuring TGase activity described in the literature (Jeoung et al. 2010; Kobayashi et al. 1996; Sokullu et al. 2008). In one of the most used methods, mTGase activity can be determined by the formation of *Z*-glutamyl-hydroxamate-glycine (a detectable iron(III) colored complex at 525 nm) using Z-Gln-Gly as the amine acceptor substrate and hydroxylamine as amine donor. A calibration curve can be constructed using L-glutamic acid γ-monohydroxamate as standard. One unit of microbial transglutaminase activity is defined as the amount of enzyme causing the formation of 1.0 μmol of hydroxamate per minute, by catalyzing the reaction between Z-Gln-Gly and hydroxylamine at pH 6.0 and 37 °C (Folk and Cole 1966; Grossowicz et al. 1950).

Additionally, several fluorescent assay procedures have been developed where it is used the increasing fluorescence intensity over time for determining enzymatic activity of transglutaminase. One of these methods consists of covalent coupling of monodansylcadaverine, catalyzed by transglutaminase to *N*,*N*-dimethylcasein using excitation wavelength 332 nm and emission wavelength 500 nm. The increase in fluorescence is proportional to the transglutaminase activity (Lorand et al. 1971).

Diversity of transglutaminases

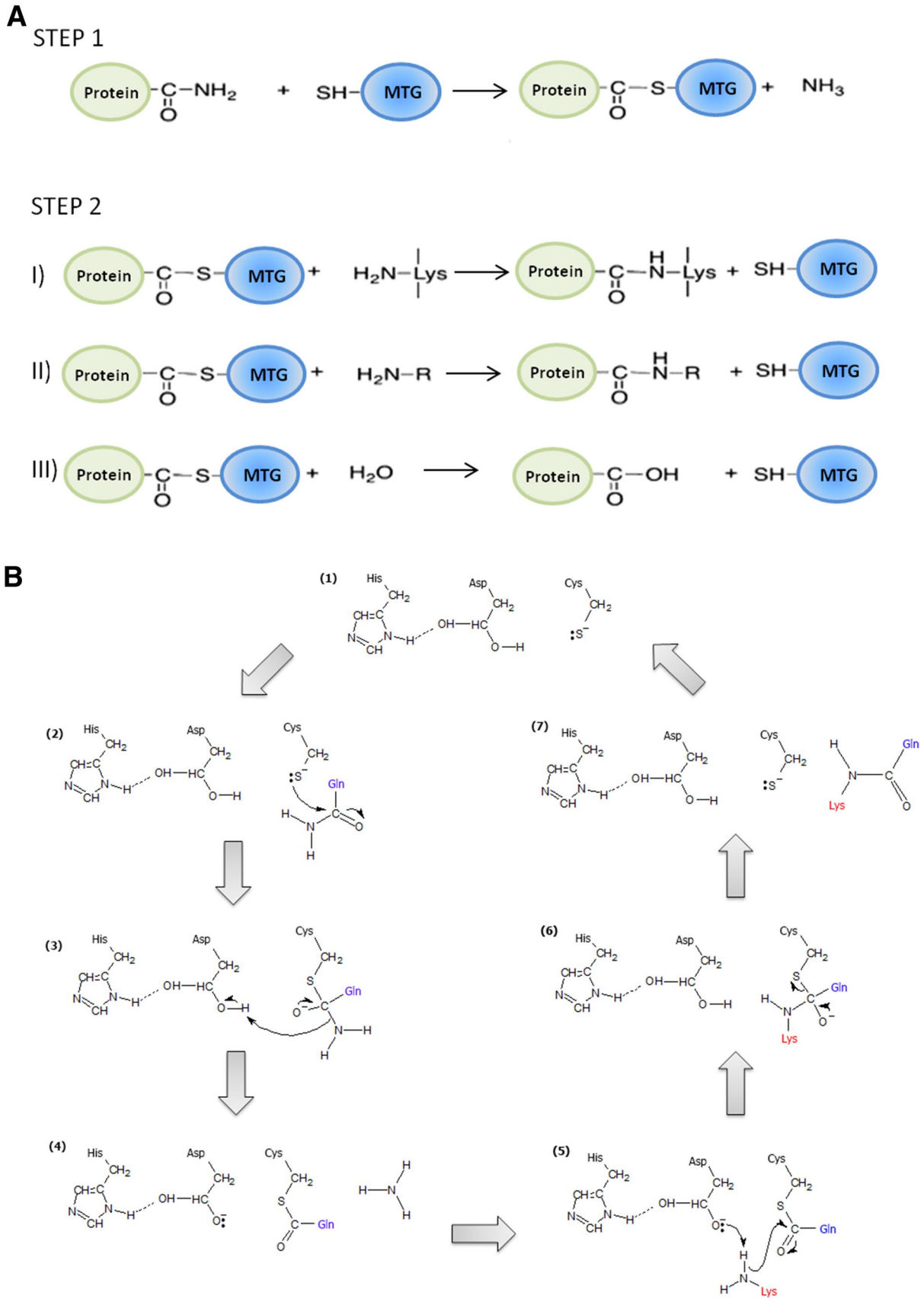
Focus will be given to most studied and used TGases, subdivided into mammalian, non-mammalian, and microbial transglutaminases.

Mammalian transglutaminases

The animal-like TGases form a large family of intracellular and extracellular enzymes with multiple functions. They are activated by calcium and produced in a zymogenic form, bound by inhibitory subunits, and/or negatively modulated by GTP/GDP or ATP (Esposito and Caputo 2004; Fernandes et al. 2015; Gundemir et al. 2012; Klöck and Khosla 2012; Lorand and Graham 2003).

In mammals, the transglutaminase family comprises nine enzymes: TG1 to TG7, factor XIII, and band 4.2, eight of which encode active enzymes, whereas one of them (erythrocyte membrane protein band 4.2) lacks enzymatic activity. Although the overall primary structure of TGase enzymes appears to be different, they are all encoded by a family of closely related genes. All mammalian TGase genes have been identified and their chromosomal positions have been mapped. Alignment of the gene products reveals a high degree of sequence similarity, with an identical amino acid sequence in the active site (Fig. 1). The nine types of TGases of this class and some of their characteristics and functions are described in succession below.

Transglutaminase 1 (TG1), also known as keratinocyte transglutaminase, is an enzyme responsible for the formation of the cornified envelope (CE), acting as a barrier against


A
STEP 1
Protein
MTG
NH3
STEP 2
I)
II)
III)
H2O
B
(1)
(2)
(3)
(4)
(5)
(6)
(7)
His
Asp
Cys
Gln
Lys

◂**Fig. 2** Reactions catalyzed by transglutaminases (TGase). **a** Scheme of the reactions in two steps acyl transfer reaction, where Step I is the formation of the intermediate acyl donor-enzyme and ammonia release and Step 2 (I) crosslinking, (II) primary amine incorporation, and (III) deamination with the free enzyme release. **b** A hypothetical catalytic mechanism of mTGase of *S. mobaraensis*. The residues of substrate proteins are Gln (substrate 1, blue) and Lys (substrate 2, red). Adapted from Kashiwagi et al. (2002)

water loss and protecting against pathogens (Aufenvenne et al. 2013; Eckert et al. 2014). Dehydration, fatal in the first weeks of life, results from autosomal recessive congenital ichthyosis caused by TG1 mutations (Cserhalmi-Friedman et al. 2002; Oji et al. 2010).

The most widely distributed and studied TGase is transglutaminase 2 (TG2), also called tissue transglutaminase. TG2 is expressed by almost all cell types in the body, being active only when bound to calcium. (Eckert et al. 2014; Grenard et al. 2001; Lorand and Graham 2003; Mehta and Eckert 2005).

The main function of TG2 is transamidation, but recent developments show that it is a multifunctional protein acting as a protein disulfide isomerase (PDI), protein kinase, scaffold protein, and even as a DNA hydrolase (Fesus and Piacentini 2002; Gundemir et al. 2012; Lorand and Graham 2003). It has been observed that expression and/or enzymatic activity is increased in several diseases, including Celiac disease, neurodegenerative diseases (e.g., Alzheimer's or Parkinson's disease), cataract formation, atherosclerosis, inflammation, fibrosis, diabetes, autoimmune diseases and in highly aggressive forms of cancer (Griffin et al. 2002; Katt et al. 2018; Lorand and Graham 2003).

Transglutaminase 3 (TG3) is also known as epidermal transglutaminase and is widely expressed in the small intestine, brain, skin, and mucosa (Eckert et al. 2014). Similar to TG1, TG3 is predominantly involved in the formation of the cornified cell envelope (critical structure for barrier function at the outermost layer of the skin epidermis) (Hitomi et al. 2001; Klöck and Khosla 2012). Studies have revealed that the down regulation of the TG3 gene is closely linked with a variety of human cancer types, including esophageal and oral squamous cell carcinoma (OSCC) (Negishi et al. 2009; Uemura et al. 2009).

Transglutaminase 4 (TG4), also known as prostate TG, is present in the prostate gland, prostatic fluids, and seminal plasma. The exact function of TG4 in humans is not well known, but some recent reports suggest a link between increased expression of TG4 and promotion of prostate cancer (Jiang and Ablin 2011; Jiang et al. 2009).

Transglutaminase 5 (TG5), also known as transglutaminase X, is a recently added member of the TGase family (Aeschlimann et al. 1998) and only its limited characterization at functional and biochemical level has been performed (Candi et al. 2004). Similarly to TG1 and TG3, TG5 is expressed in stratified squamous epithelia such as the upper layers of the epidermis, and contributes to hyperkeratosis in ichthyosis and psoriasis patients (Candi et al. 2002). TG5 inactivating mutations result in a rare pathology named Acral Peeling Skin Syndrome (APSS) in which skin peeling is strictly limited to the dorsa of the hands and feet (Cassidy et al. 2005).

Also called transglutaminase Y, Transglutaminase 6 (TG6) expression is compartmentalized in the human testes and lungs, and in the brain of mice (Eckert et al. 2014; Liu et al. 2013). Autoantibodies to TG6 were identified in immune-mediated ataxia in patients with gluten sensitivity and human carcinoma cells with neuronal characteristics also express TG6 (Thomas et al. 2013).

Transglutaminase 7 (TG7), known as transglutaminase Z, is not fully functionally-understood and few data is known about the regulation or even the function of the TG7 gene. Like TG6, TG7 expression is restricted to testes, lungs, and brain (Eckert et al. 2014). Studying the substrate preferences of TG7, it was identified a highly reactive substrate sequence for TG7 with isozyme-specificity. The knowledge of products that are possibly cross-linked by TG7 will provide more information on the physiological significance of this enzyme and diseases that may be associated with it (Kuramoto et al. 2013).

Factor XIII-A, also known as fibrin stabilizing factor, is a zymogen and becomes active by thrombin. It is a major contributor to clot formation in the final stages of coagulation. It is also important to maintain pregnancy and wound healing. In plasma, it circulates as a tetramer composed of two subunits: a subunit A (FXIII-A) and B (FXIII-B), which requires calcium and thrombin for activation (Tahlan and Ahluwalia 2014). It is produced by the liver, although it can also be found in the extracellular space and cytoplasm of various cells throughout the body (Paragh and Törőcsik 2017). Therefore, the therapeutic potential of FXIII includes invasive bacterial infections, systemic sclerosis (scleroderma), and tissue repair (in healing of venous leg or myocardial ulcers) (Dickneite et al. 2015).

Band 4.2 plays an important role in regulating cell stability and maintaining membrane integrity. It is the only TGase that has no activity because it carries a Cys → Ala substitution at the active site, which makes the protein unable to catalyze the reaction (Fig. 1). This inactive TGase is found in several tissues and cells, such as bone marrow, in erythrocytes, fetal liver, and the spleen (Eckert et al. 2014; Mariniello et al. 2008).

Non-mammalian transglutaminases

The family of TGases has been notably enlarged due to the discovery of novel isoforms in vertebrates as well as in invertebrates, plants, fungi, and microorganisms. TGase

activity was observed in different fishes, showing molecular variation among species. It was suggested that TGases may be present in eggs and skin of amphibians, in turtle shell, in epidermis, erythrocytes, and chicken gizzard (Mariniello et al. 2008; Worratao and Yongsawatdigul 2005). Transglutaminases are also present in plant tissues of soy, fava beans, beet, and orchard apple, whose activities are related to the organization of the cell wall, in antibacterial immune reactions, and in photosynthesis (Falcone et al. 1993; Kang and Cho 1996; Kashiwagi et al. 2002; Kieliszek and Misiewicz 2014; Lilley et al. 1998). It was also confirmed that more than one transglutaminase may function in one plant, or even in one organelle (Sobieszczuk-Nowicka et al. 2009). It is presented in Table 1 some important sources of non-mammalian transglutaminases.

In addition to transglutaminases from bacterial sources (mTGase), discussed below, transglutaminase activity was also found in fungi and yeasts such as *Phytophtora sojae*, *Candida albicans*, and *Saccharomyces cerevisiae* (Brunner et al. 2002; Iranzo et al. 2002; Mazáň and Farkaš 2007; Ruiz-Herrera et al. 1995).

Phytophthora sojae is a soybean pathogen that has been shown to secrete a Ca^{2+}-dependent TGase (GP42), capable of activating defense responses in plants. GP42-related proteins are only present in plant pathogenic oomycetes belonging to the order of Peronosporales (for example, *Phytophthora*, *Hyaloperonospora*, and *Pythium* spp.), and in marine *Vibrio* bacteria. Although GP42 does not share primary sequence similarities with known mammalian or bacterial TGases, it has a central region that has significant similarity to the Group A *Streptococcus* Mac-1 cysteine protease, suggesting the lateral gene transfer between bacteria and oomycetes (Del Duca et al. 2014; Reiss et al. 2011).

In the fungus *Candida albicans*, it has been suggested that the activity of TGase plays an important role in the structural organization of the cell wall possibly through the establishment of cross-links between structural glycoproteins. Activity was detected by incorporation of radioactive putrescine and most of the activity was present in the cell wall. Inhibition of growth by incorporation of cystamine (a TGase inhibitor) was also determined in other strains, demonstrating the importance of transglutaminase in these species. Cystamine also affected cell morphology, whereas the incorporation of high molecular weight proteins covalently bound to the cell wall was inhibited (Reyna-Beltrán et al. 2018; Ruiz-Herrera et al. 1995).

Likewise, in order to determine whether cross-linking of proteins by TGase would be important for *Saccharomyces cerevisiae* growth, TGase cystamine inhibitor has been used. Addition of this compound to the growth medium reduced the growth rate of *S. cerevisiae* proportionally to the concentration of the inhibitor by altering the cell morphology, indicating that TGase may be involved in the formation of the cell wall (Iranzo et al. 2002).

Table 1 Non-mammalian transglutaminases

Organism	Common name	Species	Optima temperature and pH, molecular weight	References
Fishes	Alasca Pollack	NA	85 kDa	Seki et al. (1990)
	Red sea bream	*Pagrus major*	pH 9.0–9.5, 78 kDa	Yasueda et al. (1994)
	Japanese oysters	*Crassostrea gigas*	40 °C, 84 kDa; 25 °C, 90 kDa; pH 8	Kumazawa et al. (1997)
	Tropical tilápia	*Oreochromis niloticus*	37–50 °C, pH 7.5, 85 kDa	Worratao and Yongsawatdigul (2005)
	Threadfin bream	TB, *Nemipterus sp.*	pH 7.5, 66 kDa	Piyadhammaviboon and Yongsawatdigul (2009)
	Four different fish species (Bigeye snapper, Indian oil sardine, Tilapia and Common carp)	NA	Range 73–95 kDa	Binsi and Shamasundar (2012)
Invertebrates	Shrimp	*Marsupenaeus japonicus*	85 kDa	Chen et al. (2005)
	Antarctic krill	*Euphausia superba*	0–10 °C, pH 8.0–9.0	Zhang et al. (2017)
	Crayfish	*Pacifastacus leniusculus*	4–22 °C	Sirikharin et al. (2018)
	Mythimna separata larvae	*Noctuidae, Lepidoptera*	6–42 °C, pH 7.5, 3.5 KDa	Zhang et al. (2018)
Plants	Tubers of Jerusalem artichoke	*Helianthus tuberosus*	NA	Serafini-Fracassini et al. (1988)
	Maize	*Zea mays*	NA	Villalobos et al. (2004)
	Rosemary	*Rosmarinus officinalis* L	55 °C, pH 7.0	El-Hofi et al. (2014)

NA data not available

Microbial transglutaminases

Bacterial TGases, here treated as microbial transglutaminases (mTGases), are part of an extensive transglutaminase family. Although catalyzing the same reactions, mTGases have shown to possess very little sequence similarity to any mammalian TGases (Oteng-Pabi and Keillor 2013). Although the biological function of transglutaminases in microorganisms is unclear, it is known to be a cell wall-associated enzyme and it is suggested that this enzyme may be involved in cross-linking surface proteins from air hyphae and spores of some *Streptomyces*, in addition to the formation of crosslinking between cell wall proteins in *Candida albicans* and *Saccharomyces cerevisiae* and spore coat proteins in *Bacillus subtilis* (Chater et al. 2010; Kobayashi et al. 1998; Strop 2014).

The production of mTGases were first reported by Ando et al. in 1989 for the microorganism *Streptoverticillium mobaraense*, which was later classified as *Streptomyces mobaraensis* (Ando et al. 1989; Zhang et al. 2010). In contrast to many other TGases, the microbial isoforms are not regulated by calcium or guanosine-5′-triphosphate (GTP), which makes these proteins very useful in the food industry because proteins, such as milk caseins, soybean globulins, and myosins, are sensitive and easily precipitated by Ca^{2+} (Strop 2014; Yokoyama et al. 2004). In addition, they have broader substrate specificity, lower deamidation activity, and can be low-costly mass produced by traditional fermentation technologies (Kashiwagi et al. 2002; Mariniello et al. 2008; Ohtsuka et al. 2006).

Since 1998, the enzyme has been recognized as a safe substance (GRAS) for human ingestion by the FDA (Food and Drugs Administration), making mTGases very attractive for the food industry (Gaspar and de Goes-Favoni 2015; Kieliszek and Misiewicz 2014).

After an extensive search in more than 5000 isolates of microbial origin, *Streptoverticillium* sp. strain S-8112 proved to be the first bacterium producing transglutaminase (Ando et al. 1989). From this finding, this microbial TGase has been the main source of applicable enzyme. Several studies looking for mTGase activities in microorganisms were carried out and some are listed in the Table 2, however, these activities were mostly identified in strains of *Streptomyces* and *Bacillus* genera (Jiang et al. 2017). Microbial transglutaminases of commercial interest which had their known structures will be discussed below in more detail.

Structure of transglutaminase of *Streptomyces mobaraensis*

The mTGase isolated from *Streptomyces mobaraensis* is secreted through the membrane as a zymogen (pro-mTGase) and is activated by a proteolytic processing. To activate the original zymogen, *S. mobaraensis* also secretes two proteases that are responsible for the cleavage of the N-terminal pro-peptide (Zotzel et al. 2003a, b). Its pro-region with 45-residue N-terminal is essential for efficient protein folding, secretion, and suppression of the enzymatic activity (Yurimoto et al. 2004). It folds into an L-shape and covers the active-site, blocking the substrates from accessing it, thus the site must be cleaved to allow mTGase to be rendered functional (Rachel and Pelletier 2013).

The *S. mobaraensis* mTGase forms a simple monomer showing overall dimensions of 65 × 59 × 41 Å, made up of ≈ 331 amino acids, with a molecular mass of ≈ 37 kDa and the isoelectric point at pH 8.9 (Ando et al. 1989; Kashiwagi et al. 2002). The tertiary structure of mTGase has a disk-like structure with a central groove having the active-center with a Cys-Asp-His triad, which is the key to the cross-linking efficiency (Griffin et al. 2002; Kashiwagi et al. 2002; Liu et al. 2006).

Structure of transglutaminase of *Bacillus subtilis*

A lesser-known bacterial transglutaminase from *Bacillus subtilis* was described in 1996. It has been strongly suggested that *B. subtilis* transglutaminase (bTG) form Ɛ-(Y-glutamyl)lysine bonds and it is implicated in the protection of the bacterium by causing the cross-linking of coat proteins on the surface of a spore (Kobayashi et al. 1996). The coat contributes to spore protection against several physical and chemical hazards, antagonist bactericidal enzymes, and also by playing a key role in the ability of the spore to monitor its immediate environment and to activate germination (Plácido et al. 2008).

The bTG is not related to the mammalian or other microbial transglutaminases, except for their counterparts in *Bacillus* species and some other highly related spore-formers. This enzyme functions through a catalytic dyad formed by Cys116 and Glu187 or Glu115 and the cysteine residue is required for the activity of bTG in vitro and in vivo. It also has a NlpC/P60 catalytic core, thought to represent the ancestral unit of the cysteine protease fold (Fernandes et al. 2015; Liu et al. 2014a).

In vitro, bTG is able to cross-link proteins such as BSA or α-casein (Kobayashi et al. 1998). The 20 kDa spore coat protein (GerQ) has been identified as a physiological substrate for bTG (Ragkousi and Setlow 2004; Zilhão et al. 2005). Recently, a study has allowed to screen a library of random highest affinity glutamine substrate sequences for bTG (Oteng-Pabi et al. 2018).

With a protein molecular weight of 28 kDa, bTG is ≈ 10 kDa smaller than other mTGases and shows little structural homology with the *S. mobaraensis* mTGase. The optimal temperature and pH for bTG activity are 60 °C and 8.2, respectively. Additionally, bTG is expressed as a

Table 2 Microorganisms producing transglutaminases

Species	Strain	Conditions of cultivation	Activity	Optima temperature and pH, and molecular weight	References
Streptoverticillium mobaraensis	S-SI12	Polypepton, glucose, K_2HPO_4, $MgSO_4$ at 30 °C for 48 h	2.5 U/mL	50 °C, pH 6, 40 kDa	Ando et al. (1989)
	S-SI12	Polypeptone, yeast extract, K_2HPO_4, $MgSO_4$, potato starch, glucose, pH 7.0 at 30 °C for 9 -11 days	2.1 U/mL	50 °C, pH 6, 40 kDa	Gerber et al. (1994) and Ando et al. (1989)
	CBS 20778	Soluble starch, peptone, $MgSO_4$, KH_2PO_4, K_2HPO_4, yeast extract and was supplemented with certain amino acids, pH 6.5 at 28 °C for 48 h	1.8 U/mL	NA	Zhu et al. (1998)
	CBS 20778 (WSH-Z2)	Starch, peptone, yeast extract, MgSO4, K_2HPO_4, KH_2PO_4, pH7.0 at 30 °C for 7 days	2.9 U/mL	30 °C	Zheng et al. (2002)
	CBS 20778 (WSH-Z2)	Starch, glucose, peptone, yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4, pH 6.8 at 30 °C for 48 h	3.3 U/mL	NA	Yan et al. (2005)
Streptoverticillium sp.	s-8112	NA	NA	37 kDa	Kanaji et al. (1993)
Streptoverticillium cinnamoneum	CBS 683.68	Soya peptones, casein, glycerol, $MgSO_4$, KH_2PO_4, Na_2HPO_4, yeast extract, and oligoelements ($FeSO_4$, $ZnSO_4$, $MnSO_4$) at 28 °C for 120 h	0.3 U/mL	37 °C, pH 6	Junqua et al. (1997)
Streptoverticillium ladakanum	ATCC 27441	Glycerol, yeast extract, K_2HPO_4, $MgSO_4$, pH 7.0 at 28 °C for 4 days	NA	40 °C, pH 5.5	Ho et al. (2000)
	NRRL-3191	Xylose, yeast extract, peptone, $MgSO_4$, KH_2PO_4, Na_2HPO_4 and sodium caseinate at 26 °C for 120 h	0.3 U/mL	NA	Téllez-Luis et al. (2004)
Streptomyces lydicus	NA	NA	2.2 IU/mg	37 °C, pH 6, 37 kDa	Langston et al. (2007)
Streptomyces nigrescens	NA	NA	0.6 IU/mg	37 ºC, pH 8, 36 kDa	Langston et al. (2007)
Streptomyces hachijoensis	NA	NA	1.9 IU/mg	35 kDa	Langston et al. (2007)
Streptomyces cinnamoneus	NA	NA	0.2 IU/mg	39 kDa	Langston et al. (2007)
Streptomyces hygroscopicus	WSH03-13	Starch, glucose, glycerin, peptone, soybean powder, yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4 and $CaCO_3$, pH 6.5 at 32 °C for 42 h	NA	37–45 °C, pH 6–7, 38 kDa	Cui et al. (2007)
Streptomyces mobaraensis	NA	NA	3.9 IU/mg	37 °C, pH 8, 37 kDa	Langston et al. (2007)
	NRRL B-3729	Wheat bran–soybean meal mixture (9:1), KH_2PO_4, NH_4NO_3, $MgSO_4$, NaCl, $CoCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, pH 6 at 30 °C for 7 days	800 IU/mg	50 °C, pH 7, 37 kDa	Nagy and Szakacs (2008)
Streptomyces platensis	NA	NA	1.5 IU/mg	37 °C, pH 7–8, 38 kDa	Langston et al. (2007)
	NRRL 2364	Liver kidney bean, KH_2PO_4, NH_4NO_3, $MgSO_4$, NaCl, $CoCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, pH 6 at 30 °C for 7 days	5100 IU/mg	45 °C, pH 8, 37 kDa	Nagy and Szakacs (2008)
Streptomyces paucisporogenes	ATCC 12596	Liver kidney bean, KH_2PO_4, NH_4NO_3, $MgSO_4$, NaCl, $CoCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, pH 6 at 30 °C for 4 days	4200 IU/mg	45 °C, pH 8, 37 kDa	Nagy and Szakacs (2008)
Streptomyces sp.	CBMAI 837	Soybean flour, potato starch, glucose, peptone, KH_2PO_4, and $MgSO_4$ at 30 °C for 5 days	0.4 U/mL	35–40 °C, pH 6–6.5, 45 kDa	Macedo et al. (2011)

Table 2 (continued)

Species	Strain	Conditions of cultivation	Activity	Optima temperature and pH, and molecular weight	References
Bacillus subtilis	AJ12866	Schaeffer's sporulation medium (SSM) at 37 °C for 18 h	NA	50 °C, pH 8, 23 kDa	Kobayashi et al. (1998)
	AJ1307	Schaeffer's sporulation medium (SSM) at 37 °C for 9.5 h	NA	60 °C, pH 8.2, 29 kDa	Suzuki et al. (2000)
Bacillus circulans	BL32	SLC medium (soluble starch, peptone, yeast extract, $MgSO_4$, K_2HPO_4 and KH_2PO_4), pH 7 at 30 °C for 24 h	0.69 U/mL	NA	de Barros Soares et al. (2003a)
	BL32	SLC medium (soluble starch, peptone, yeast extract, $MgSO_4$, K_2HPO_4 and KH_2PO_4), pH 6.5 at 30 °C for 240 h	NA	47 °C, pH 7, 45 kDa	De Barros Soares et al. (2003b)
	BL32	Optimized medium (glycerol, sucrose, peptone, tryptone, Na_2HPO_4, $MgSO_4$ and $FeSO_4$) at 30 °C for 240 h	0.31 U/mL	NA	Souza et al. (2006)

NA data not available

mature peptide, unlike mTGase, which exists as pro-enzyme (Oteng-Pabi et al. 2018).

Recombinant microbial transglutaminases

The industrial enzyme market was evaluated at around US$ 6.1 billion in 2017 and it is expected to reach US$ 8.5 billion by 2022 (Ferrer et al. 2015). Much of this production is concentrated in enzymes for the food industry. More than 55 different enzyme products are used in the food processing industry and the number is permanently increasing, related to the discovery of new food enzymes (Fernandes 2010).

Aiming to develop innovative, sustainable, and economically competitive production processes, there is an increasing need for new, more versatile and improved enzymes. Novel researches in molecular genetics and cell biology over the past four decades has reconfigured enzyme production. The majority of industrial enzymes are already coming from recombinant sources produced in bacteria, fungi and yeasts (Adrio and Demain 2014; Olempska-Beer et al. 2006). Several studies have been focused in the gene expression of mTGase in *Streptomyces lividans*, *Corynebacterium glutamicum*, *Yarrowia lipolytica*, *Streptomyces platensis*, and *Escherichia coli*, as described below (Date et al. 2004; Lin et al. 2004, 2006a; Liu et al. 2015; Mu et al. 2018b; Rickert et al. 2015; Salis et al. 2015; Washizu et al. 1994). Table 3 shows some of the recombinant transglutaminases treated in this section, showing details of construction and culture information.

Streptomyces lividans

One of the earliest works on recombinant mTGase expression was presented in 1994 when the *Streptoverticillium mobaraense* gene was cloned and expressed in *Streptomyces lividans* 3131 under the control of a tyrosinase promoter, yielding an active and mature enzyme. However, the secretion level of mTGase in *S. lividans* 3131 was very low, less than 0.1 mg/L, not suitable for industrial applications (Washizu et al. 1994).

The gene mTGase from *Streptoverticillium ladakanum* B1 was cloned and expressed in *Streptomyces lividans* JT46 using an endogenous promoter. The revealed result of immunoblotting of SDS-PAGE indicated that the recombinant mTGase was not correctly processed (Lin et al. 2004). Following ahead with this study, the same group of researchers cloned and expressed the gene of mTGase of *Streptomyces platensis* M5218 in *Streptomyces lividans* JT46, with a 3.3-fold increase in enzyme activity in relation to that from the wild *S. platensis* M5218 strain (Lin et al. 2006b).

The mTGase obtained from *Streptomyces hygroscopicus* WSH03-13 was cloned into plasmid pIJ86 and has been

Table 3 Recombinants mTGases

Recipient strains	TGase gene donor	Expression construction	Substrate induction	Conditions of cultivation	Activity/yield/specific productivity	References
Streptomyces lividans 3131-TS	*Streptoverticillium mobaraense* S-8112	pIJ702-derived plasmids, tyrosinase promoter	20 µg/ml thiostrepton and 40 µg/mL tyrosine	30 °C for 5 day	0.1 mg/L	Washizu et al. (1994)
Streptomyces lividans JT46	*Streptomyces platensis* M5218	pIJ702 and pAE053	20 µg/mL thiostrepton and 40 µg/mL tyrosine	R2YE agar or liquid medium (glucose, tryptton peptone, KH_2PO_4, $MgSO_4$, yeast extract, and glycine), 30 °C for 3 days	2.2 U/mL	Lin et al. (2006b)
Streptomyces lividans TK24	*Streptomyces hygroscopicus* WSH03-13	pIJ86	Endogenous promoter or its partially deleted (50 µg/mL apramycine)	R2YE agar or liquid medium (glycerol, peptone, yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4, and $CaCl_2$), 30 °C and 200 rpm for 2–3 days	5.73 U/mL and 0.14 U/mL/h	Liu et al. (2016)
Corynebacterium glutamicum ATCC 13869	*Streptoverticillium mobaraense* IFO13819	Fusion gene is controlled by the *cspB* promoter	Signal peptides	MMTG medium (glucose, $MgSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, $FeSO_4$, $MnSO_4$, $CaCO_3$, thiamine, hydrochloride, biotin, and DL-methionine), pH 7.5 at 30 °C for 140 h	142 mg/L	Kikuchi et al. (2003)
Corynebacterium glutamicum ATCC 13032	*Streptomyces mobaraense* CICC 11018	pXMJ19, tac-M promoter	Signal peptide ΔS0949, IPTG inducer	MMTG medium at 30 °C for 12 h, followed by an additional 40 h cultivation after induction	6.7 U/mL	Liu et al. (2014b)
Yarrowia lipolytica Po1h	*Streptomyces hygroscopicus* WSH03-13	pINA1296 (a pBR322-based mono-copy integrative vector) and pINA1297 (an auto-cloning multi-copy integrative vector)	NA	Modified PPB medium (glucose, yeast extract, NH4Cl, KH2PO4, MgSO4, and thiamine), pH 6.0, at 28 °C, 200 rpm for 5 days	5.3 U/mL	Liu et al. (2015)
Escherichia coli Rosetta (DE3)	*Streptomyces hygroscopicus* H197	pET32a+	IPTG	LB medium containing 100 µg/mL ampicillin and incubated at 37 °C or 25 °C for 8 h	0.69 U/mg	Wan et al. (2017)
Escherichia coli BL21 (DE3)	*Streptomyces mobaraensis*	pET22b	NA	Terrific Broth containing 25 µg/mL kanamycin at 25 °C for 60 h	120 mg/L or 1 U/mL	Javitt et al. (2017)

Table 3 (continued)

Recipient strains	TGase gene donor	Expression construction	Substrate induction	Conditions of cultivation	Activity/yield/specific productivity	References
Pichia pastoris GS115	*Zea mays*	pPIC9K	Methanol (5%, v/v) once every 24 h	BMMY medium (except methanol instead of glycerol) at 28 °C at 250 rpm for 96 h	4.4 mg/L and 0.889 U/mg	Li et al. (2013)
Pichia pastoris GS115	*Streptomyces fradiae*	pPIC9K	Methanol (5%, v/v) once every 24 h	BMMY medium (yeast extract, peptone, 0.1 M potassium phosphate, yeast nitrogenous base without amino acids, methanol, and biotin) pH 6.0, at 30 °C for 4 days	0.70 U/mL	Yang and Zhang (2019)

NA data not available

expressed in *S. lividans* TK24. Based on deletion analysis, it was identified a negative element in the mTGase putative promoter, and the deletion of this element increased the mTGase production by up to 81.3%. Combining optimization of the gene codons and deletion of the negative promoter element, the recombinant *S. lividans* TK24 produced mTGase activities of up to 5.73 U/mL and a maximum productivity of 0.14 U/mL/h (Liu et al. 2016).

Corynebacterium glutamicum

It has been shown that *C. glutamicum* ATCC 13869 is efficiently able to secrete the pro-mTGase from *S. mobaraense* IFO13819, when it is coupled to signal peptides derived from the cell surface proteins of *Corynebacterium*. Moreover, when a protease (SAM-P45) from *Streptomyces albogriseolus* is co-secreted by *C. glutamicum,* the pro-domain is then processed, and the enzyme is converted into active-form mTGase. The maximum yield of the active form was 142 mg/L (Kikuchi et al. 2003). Replacing the pro-region of transglutaminases of *Streptomyces mobaraensis* by the pro-region of transglutaminases of *Streptomyces cinnamoneus* for the production of mTGase in *C. glutamicum*, increased secretion of mTGase by 23% compared to that using the native pro-region (Date et al. 2004).

Screening for the secretion of pro-mTGase, 16 strains of coryneform bacteria were tested and it was discovered that most of them secreted pro-transglutaminase. The *Corynebacterium ammoniagenes* ATCC6872 was the best producing strain, with about 2.5 g/L pro-transglutaminase over a 71 h culture in a jar fermentor (Itaya and Kikuchi 2008).

In order to improve mTGase secretion on a recombinant *Corynebacterium glutamicum* strain, it was performed a metabolic flux analysis involving ^{13}C isotope-labeling experiments (^{13}C-MFA). The strategy for enhancing mTGase secretion was developed and its effectiveness was confirmed. It was also checked that the increase in the flux to the tricarboxylic acid (TCA) cycle might result in an increase in the NADH/NAD$^+$ ratio, which is believed to be one of the reasons for the decrease in mTGase yields. In addition, with the aim of decreasing the NADH/NAD$^+$ ratio, lactate production was increased by raising the pH level in the culture, successfully increasing mTGase production (Umakoshi et al. 2011).

Further improvements on mTGase production in the heterologous host *C. glutamicum* could be achieved by the use of more powerful promoters. The mTGase secreted by *Streptomyces mobaraense*, expressed in *Corynebacterium glutamicum* ATCC, was optimized by the promoter exchange tac for tac-M, with mTGase activity of 5.2 U/mL for the first and 6.7 U/mL for the second construct (Liu et al. 2014b).

Yarrowia lipolytica

In one remarkable research, *Streptomyces hygroscopicus* pro-mTGase was efficiently expressed in *Yarrowia lipolytica*, without the need for antibiotic markers. The gene was cloned into integrative vectors monocopy and multicopy. A recombinant promoter drove the obtained expression and secretion using a XPR2 pre-sequence as a signal peptide. The highest yield of extracellular pro-mTGase was achieved by the recombinant multicopy construct, with 5.3 U/mL of mTGase. In order to improve mTGase properties, asparagines in two predicted Asn-linked glycosylation sites (Asn160 and Asn355) of pro-mTGase were mutated to glutamines. Thereby, the mTGase yield of variant was increased to 35.3 U/mL by using a glycerol feeding strategy in a 3 L fermenter (Liu et al. 2015).

Escherichia coli

Escherichia coli has been by far the most important bacterium for cloning research. One of the earliest cloning of mTGase was performed in 1994, when mTGase from *Streptoverticillium* was chemically synthesized and inserted in the vector pIN-III with the ompA signal peptide and expressed in *E. coli*. Although the induced gene product was identical to the native enzyme, the activity was low (Takehana et al. 1994). In another attempt to overexpress mTGase, the gene from *Streptoverticillium* was chemically synthesized by fusion to a bacteriophage T7 gene 10 leader peptide (260 amino acids), using an inducible expression vector. The mTGase gene was expressed producing inclusion protein bodies in the *E. coli* cytoplasm. It was necessary to solubilize the protein with subsequent proteolytic cleavage to achieve enzyme activity of mTGase (Kawai et al. 1997).

Using the pro-domain engineering, it was possible to achieve good expression levels of soluble and fully active mTGase from *Streptomyces mobarensis* in the cytoplasm of *E. coli*. Through an alanine-scan of the mTGase pro-domain and the insertion of the 3C protease cleavage site, it was possible to achieve expression levels of 30 to 75 mg/L of fully active mTGase (Rickert et al. 2015).

mTGase from *Streptomyces hygroscopicus* H197 was mutated by cleaving a specific 84 bp fragment and expressed in plasmid pET32a + in *E. coli* Rosetta cell, aiming to achieve high stabilities and activities. The purified mutant showed 0.22 U/mg and 0.69 U/mg mTGase activities before and after activated by trypsin, respectively, compared to the wild mTGase 0.16 U/mg and 0.54 U/mg activity under the same conditions (Wan et al. 2017).

The active mTGase expression of *S. mobaraensis* in *E. coli* by one constitutive system was devised without the use of a downstream proteolytic cleavage processing, obtained by constructing a synthetic operon with a pro-domain encoding gene and a gene encoding the mTGase thermostable variant, both sequences paired with a previous PelB secretory sequence. The expressed products of this investigation were segregated in the periplasm, making easier the correct folding of the enzymes and reducing the formation of inclusion bodies (Javitt et al. 2017).

Developing novel designs of enzymes took two different types of synthetic components to be simultaneously incorporated into mTGase from *S. mobaraense,* through the engineering of thermostable variants and expressed in *E. coli*. The first amino acid, 3-chloro-l-tyrosine, was incorporated into mTGase in response to in-frame UAG codons to impute an increase thermostability of the enzyme. With this, the half-life was 5.1-fold longer than that of the wild-type enzyme at 60 °C. In sequence, this mTGase variant was further modified by incorporating the α-hydroxy acid analogue of NƐ-allyloxycarbonyl-l-lysine (AlocKOH), specified by the AGG codon, at the end of the N-terminal inhibitory peptide, which led to the overall stabilization of the enzyme (Ohtake et al. 2018).

Pichia pastoris

The first transglutaminase cloned in *Pichia pastoris* was the transglutaminase from *Zea mays*. This TGase was first expressed in *E. coli*, but the recombinant TGase was mainly found as inclusion bodies and the activity of the obtained protein was low (Carvajal et al. 2010). Researchers have since shift to clone *Z. mays* TGase sequences in *P. pastoris* using well-characterized yeast expression vectors, producing a soluble protein. Showing a fast growth rate, when coupled with high cell-density fermentation for secreting proteins that can be purified from the culture medium, *P. pastoris* is a promising cloning system for basic laboratory research and for industrial manufacturing (Weinacker et al. 2013). The expressing of *Z. mays* TGase in *P. pastoris* GS115, using the vector pPIC9K produced specific activities of 0.321 U/mg and mass yields of 4.4 mg/L (Li et al. 2014). Modification of codon bias of *P. pastoris* optimized TGase production and specific activities reached 0.89 U/mg (Li et al. 2013). By applying the Plackett–Burman (P–B) design and the response surface methodology (RSM) using the same expression model, authors found 1.1 U/mL of TGase activity and mass yields of 7.6 mg/L of TGase (Li et al. 2017).

In a recent study, the TGase gene from *Streptomyces fradiae* was cloned and expressed in *Pichia pastoris* GS115, showing enzyme activity of approximately 0.70 U/mL, proving that mTGase can be heterogeneously expressed (Yang and Zhang 2019).

Under the control of the constitutive GAP promoter using *Pichia pastoris*, Türkanoğlu Özçelik et al. (2019) expressed the microbial pro transglutaminase (pro-MTGase) from *Streptomyces mobaraensis*. The obtained enzymatic activity

was calculated as 37,640 U/L for large-scale production (Türkanoğlu Özçelik et al. 2019).

Bacillus subtilis

Bacillus subtilis is a Gram-positive, non-pathogenic strain and is generally recognized as safe (GRAS). Its physiology is well investigated, and, for its genetic manipulation, a variety of tools and vectors are available. Moreover, *B. subtilis* does not produce endotoxins, which is an advantage in downstream processing (de Boer Sietske and Diderichsen 1991; Schallmey et al. 2004). Two different secretion systems were constructed for cloning and secretion of mTGase from *S. mobaraensis* in *B. subtilis*. One involves inducible expression, under the control of the promoter P lac and the other containing a constitutive expression under the control of the promoter P hpaII. With peptides signals fused to the mTGase gene, it was possible to secrete pro-mTGase into the medium. After proteolysis of the pro-domain with trypsin, the concentrations of transglutaminase were: 63 mg/L for the constitutive system and 54 mg/L for the inducible system, showing enzymatic activities as high as 29 U/mg (Mu et al. 2018a).

Conclusion

Transglutaminases remain as one of the most important and complex family of enzymes, possessing varied structures and functions in mammalians, non-mammalian eukaryotes, and in bacteria. In recent years, several studies have been performed in relation to gene expression of transglutaminases, in order to gain versatility and to obtain more stable enzymes for broader industrial applications. Reduction of costs of production are essential aiming their application on a larger scale in industrial sectors such as in food production and biotechnological products.

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Compliance with ethical standards

Conflict of interest All authors of this research declare to have no conflict of interest.

Research involving human and animal participants Research was conducted without using human or animal experimentation.

Informed consent No informed consent was necessary to conduct this research.

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