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BIOINFORMATIC ANALYSIS AND PURIFICATION OF GLUTATHIONE TRANSFERASE (GST) from *Pseudomonas* sp. UW4

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ABSTRACT

The study aimed at identifying and purifying cytosolic glutathione transferase isoforms expressed in *Pseudomonas* sp. UW4. Search at UniProt (https://www.uniprot.org/uniprot/), has indicated that there were 20 genes encoding putative glutathione transferases for the microorganism. The molecular weights of the isoforms ranged from 17.6 to 34.06 kDa. SDS-polyacrylamide gel electrophoresis revealed that the GST purified using Sulfobromophthalein-glutathione (BSP) affinity column, resolved into a single band with a low molecular weight (MW) of 16 kDa with the pI value of 6.0. Purified GST was reactive towards ethacrynic acid, 1-chloro-2,4-dinitrobenzene, cumene hydroxide, and hydrogen peroxide, but no detectable activity with Trans-2-octenal, hepta-2,4-dienal and Trans-4-phenyl-3-butene-2-one. This has proven that putative GST possessed peroxidase activity and proposed to be similar to PputUW4_00801 (putative glutathione S-transferase) of *Pseudomonas* sp. UW4 according to its estimated molecular weight and the pI values obtained experimentally.

Key words: Affinity chromatography, glutathione transferases, *Pseudomonas* sp. UW4

INTRODUCTION

Glutathione S-transferases (GSTs) are a diverse superfamily of enzymes that are found naturally in various organisms such as microorganisms, insects, plants, fish, and mammals (Philip *et al*., 2001; Shehu *et al*., 2019). These enzymes have various types of catalytic activities mainly in the cellular detoxification process.

During the catalytic activities, GSTs enzymes will lower the *p*Ka of the sulfhydryl group of reduced tripeptide glutathione (GSH) from pH 9.0 in an aqueous solution to about pH 6.5 when GSH is bound in the active site (Pakorn *et al*., 2005). The sulfur atom of the GSH will then bind with endobiotic and xenobiotic electrophilic substrates through a thioether bond (Philip *et al*., 2001). Other than their detoxification role, GSTs are multifunctional enzymes as they also involve in many other processes such as reductive maintenance of thiolated proteins prostaglandin synthesis, intracellular support of different types of hydrophobic ligands, sequestering of carcinogens, involving pathway of intracellular signal transduction. (Shi *et al*., 2014).

According to the proteins sequence and structure form, there are at least four major superfamilies GSTs being classified which are cytosolic GSTs, mitochondrial GSTs, microsomal GSTs, and fosfomycin resistance protein (Skopelitou *et al*., 2012). GSTs also called canonical GSTs (cGSTs) as they exhibit in homodimer or heterodimeric folds. Those in the same classes have more than 40% similarity in their amino acid sequences. The amino acid sequences between the classes are reduced to less than 25% similarity. Besides that, the primary structures at the N-terminus active site are conserved within the classes. The tyrosine, serine, or cysteine residue are commonly found in the active site of the N-terminal which forms a bonding with the thiol group GSH (Oakley, 2011).

Each of the classes or subfamilies consists of around five different homogenous polypeptide chains which have a similarity of more than 90%. Therefore, each class of the respective families can be classified using definite gene structures and chromosomal localizations as amino acid residues at positions 60 and 80 are well conserved (Sheehan *et al*., 2001). Studying both specific expressions of GSTs and immunological interactions can be identified using immunoblotting.

Cytosolic GSTs have formed the largest superfamily and most of them existed in dimers form. By comparing the substrate specificity and primary structure, these enzymes are classified into 13 sub-classes which are alpha, beta, delta, epsilon, theta, mu, nu, omega, pi, sigma, tau, phi, and zeta (Oakley, 2011). The alpha, mu, pi, and theta class genes have differences in size and intron or exon

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structure. However, there are a few classes of this superfamily, called beta (prokaryotes), chi (bacteria & insects), delta, epsilon, lambda, phi, and tau (plants) are restricted to non-mammalians species (Pandey *et al*., 2015). Plant GSTs belong to phi, tau, theta, zeta and lambda classes. Both theta and zeta classes can be found in animals whereas the sigma and theta classes are mainly exhibited in invertebrate animals (Nebert *et al*., 2004).

The mammalian cytosolic GSTs can be further classified into alpha, mu, omega, pi and theta, and zeta classes (Pandey *et al*., 2015) after comparing their nucleotide sequence, immunological identity, substrate and inhibitor specificity, kinetic and structural properties. This classification was confirmed as protein sequence data. Within each of the classes, they have about 60 to 90% similarity in their sequence and around 30% between classes.

The mitochondrial GSTs (kappa class GSTs) are soluble dimeric proteins identified from mammalian mitochondria. Mitochondrial GSTs and cytosolic GSTs are exhibited in dimer folds and the heterodimers of cytosolic GSTs have been proven to contain the chains fit the same classes. These have shown both classes have an evolutionary relationship (Allocati *et al*., 2009).

The microsomal GSTs are known as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). These enzymes are classified into four subgroups (I-IV) based on their protein sequences (Bresell *et al*., 2005). Between each of the subgroups, the similarity of the protein sequences is less than 20%. Some of the MAPEGs (for example GSTM1, MAPEG isoenzymes found in humans) shared common characteristics with cytosolic and mitochondrial GSTs which can catalyze the conjugation of GSH to endobiotic and xenobiotic electrophilic substrates. Most of the MAPEG members contribute to the production of leukotrienes and prostanoids, and lipid signaling compounds in living things. Bacterial MAPEG proteins have been divided into two families (Oakley, 2011). The representative of each group is *E. coli* and *Synechocystic* sp. protein. All three families mentioned above are found in prokaryotes however the fourth subfamily is present in bacteria only.

The conservation of the structural features was observed across all the classes of GST enzymes to understand their different capability in function and sequence divergence of GST enzymes. All the identified GSTs from different classes have two domains with the same protein folding. Most of the N-terminal domain consists of the GSH binding site and adopts a βαβαββα unit, whereas the C-terminal domain is in charge of the binding of hydrophobic compounds (Oakley *et al*. 2001). From past research, nearly all the highly conserved residues that are found in alpha, mu, and pi classes are not found in bacterial and theta class GSTs (Vuilleumier, 1997).

Compared to eukaryotes, information on prokaryotic GSTs was inadequate but current research showed the finding of GSTs in different types of aerobic bacteria. The first research on the presence of bacterial GSTs was found by Takashi Shishido who discovered the GST activity in *Escherichia coli*. Bacterial GSTs have recently been discovered and grouped into different classes based on their functional versatility. However, some of the bacterial GSTs of the same class express differently in catalytic function (Skopelitou *et al*., 2012).

Bacterial GSTs serve an important role in many types of chemical transformations and detoxification. Due to their functional versatility and sequence variability, bacterial GSTs are involved in biodegradation and bioremediation such as the biodegradation of toxic pollutants, protection against chemical and oxidative stresses, cellular protection from reactive oxygen species, degradation of several monocyclic aromatic compounds, and antimicrobial drug resistance (Allocati *et al*., 2009).

The plant growth-promoting bacteria *(PGPB) Pseudomonas* sp. UW4 was obtained and isolated from the rhizosphere of common reeds growing on the campus of the University of Waterloo, Ontario, Canada. *Pseudomonas* sp. UW4 allows plants to grow and withstand different types of environmental stresses (Duan *et al*., 2013). Therefore, it would be remarkable to study the contribution of GSTs to the resistance to environmental stresses. This project serves as a preliminary study of identifying putative GSTs of *Pseudomonas* sp. UW4 through bioinformatic analysis, purifying expressed GSTs from *Pseudomonas* sp. UW4 and characterizing the purified GSTs.

MATERIALS AND METHODS

Collection of *Pseudomonas* sp. UW4 sample

Pseudomonas sp. UW4 pure culture was obtained from the University of Waterloo.

Protein extraction from *Pseudomonas* sp. UW4

A single colony was picked from the tryptic soy agar plate and grown aerobically in the 100 mL sterilized tryptic soy broth containing 100 µg/mL ampicillin sodium for 24 h at 30 °C, followed by sub-cultured 50 mL into 1 L of sterilized tryptic soy broth containing 100 µg/mL ampicillin sodium respectively and incubated for another 48 h at 30 °C. The culture was then centrifuged under 6000 rpm, for 20 min at 4 °C. The collected cell pellet was washed and well mixed in 5 mL of cold 25 mM of sodium phosphate buffer, pH 7.4. All the samples were combined and centrifuged under 6000 rpm, for 45 min at 4 °C. 9.9 mL of cold homogenizing buffer (25 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.1 mM DTT, 0.1 mM PTU, and protease inhibitor, was

added into the cell pellet and well mixed. 100 µL of 1 mg/mL muramidase (Fluka Analytical – 96381 U/mg) was added and left for 45 min at room temperature. The suspended cells were disrupted using WISE-TIS Homogenizer HD-15G. The particulate material was removed after being centrifuged at 10500 ×*g* for 45 min at 4 °C (DI ILIO *et al*., 1988). The supernatant was collected and applied to an affinity column which was pre-equilibrated with eluting buffer.

Purification of GSTs by using affinity chromatography

Fast protein liquid chromatography (FPLC) was washed with 20% ethanol, and distilled water, followed by eluting buffer (cold 25 mM of sodium phosphate buffer, pH 7.4). Sulfobromophthalein-glutathione (BSP) matrix column was used to trap and purify GSTs from the sample. The packed BSP column was then connected to AKTA Purifier™. The column was washed and equilibrated using an eluting buffer at a flow rate of 0.3 mL/min. The sample was injected into the affinity chromatography with a flow rate of 0.3 mL/min. Next, the sample was eluted with eluting buffer containing 0.5 M potassium chloride to remove all the unwanted protein that bound to the BSP matrix, and the flow rate was set at 0.50 mL/min. The flow-through was not collected. BSP column with bound GSTs was eventually eluted with eluting buffer containing 2 mM BSP and stored at 4 °C. While collecting, the DEAE Sepharose fast flow column was attached below the BSP matrix column and both connected columns were pre-equilibrated with eluting buffer. All the purified protein was stored at 4 °C for kinetic and substrate specificity determination or stored at −20 °C until further analysis. Protein determination was determined using the Bradford assay (Bradford, 1976).

Molecular weight determination by using SDS-PAGE

Both resolving gel and stacking gel were prepared in 12% and 4% respectively. 0.8 µL marker and 20 µL sample were loaded into the well respectively. The electrophoresis was run at 120 V under room temperature. BenchMark™ was used as a molecular size marker. The Vorum silver stain method was used to stain the gel (Mortz *et al*., 2001).

Isoelectrofocusing

Isoelectrofocusing was performed to determine the pI value of the sample protein. Novex IEF Gels and the XCell SureLock Mini-Cell were used. Two running buffers, IEF Cathode Buffer (Invitrogen) and IEF Anode Buffer (Invitrogen) were prepared and used. The system was set at 100 Volt for the first hour, then 200 Volt for the following hour then 500 Volt for the last 30 min. The plate was removed from the electrophoresis system once it stopped running. The IEF gel was first fixed in 12% TCA for 30 min before staining with silver. The gel was checked using Image Scanner III (GE Healthcare) and analyzed with Image Master Software.

Substrate specificities

Common substrates for GST essays such as 1-Chloro- 2,4-dinitrobenzene (CDNB), ethacrynic acid (EA), sulfobromophthalein (BSP), p-nitrobenzyl chloride (NBC), trans-4- phenyl-3-buten-2-one (PBO), cumene hydroperoxide and hydrogen peroxide were used to determine the enzymatic activities (Wendel, 1981).

Bioinformatic analysis

Using bioinformatic techniques, GST sequences were identified in publicly accessible databases (https://www.uniprot.org/uniprot/), and all of the organism's putative GSTs sequences were identified. UPGMA method was used to suggest the evolutional relationship and history. The phylogenetic tree is drawn to scale, and the branch lengths were indicated by the evolutionary distances used. The evolutionary distances were calculated using the Poisson correction method. The study retrieved 38 other Glutathione transferases sequences from different classes of the isoforms from UniProt ((https://www.uniprot.org/uniprot/), and the evolutionary analyses were conducted in MEGA7 software with the inclusion of the identified putative GSTs of the studied organism.

RESULTS AND DISCUSSION

The crude supernatant was collected from the lysed culture and examined the activity on EA and CDNB. The results suggest the presence of GSTs in this isolate. Three types of affinity columns were then used to purify GSTs from the crude protein. Many bands were found on the SDS-PAGE after purification using the GSTrap™ column (Figure 1b) and DNP glutathione affinity column chromatography (Figure 1c) respectively which recommended that both columns were not effective to trap GST from the crude protein. However, after purification using sulfobromophthalein-glutathione (BSP) affinity column chromatography, a single band was visualized on SDS-PAGE. The molecular weight (MW) was estimated to be 16 kDa (Figure 1a) somehow it was lower as compared to the range of GSTs molecular weights. A further vertical isoelectric focusing was performed and revealed that the band only existed in a single isoform of GST with a pI value of approximately 6.0 (Figure 2).

Table 1 indicates that purified putative GST had the highest affinity towards EA compared to the other substrates. According to early studies, this has similar behavior with Pi (π) class GSTs (Yang *et al*.,

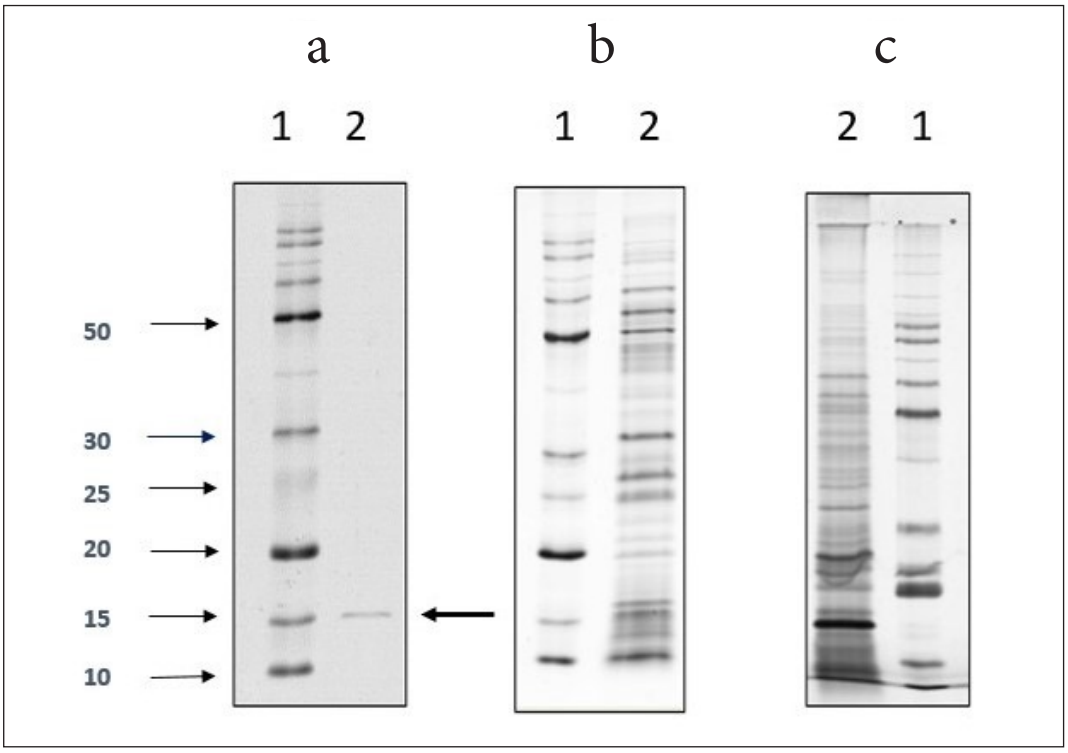

Fig. 1. SDS PAGE of purified protein from *Pseudomonas* sp. UW4 using BSP affinity column. (a) Lane 1 shows the BenchMark™ marker (Invitrogen). Lane 2 shows the presence of single band GST with MW estimated at 16 kDa (10 µg) (indicated by arrow). (b) SDS PAGE of purified protein from *Pseudomonas* sp. UW4 using GSTrap™ column. Lane 1 shows the BenchMark™ marker (Invitrogen). Lane 2 shows a purified GST sample. (c) SDS PAGE of purified protein from *Pseudomonas* sp. UW4 using GSTrap™ column. Lane 1 shows the BenchMark™ marker (Invitrogen). Lane 2 shows a purified GST sample. Gels were stained with silver.

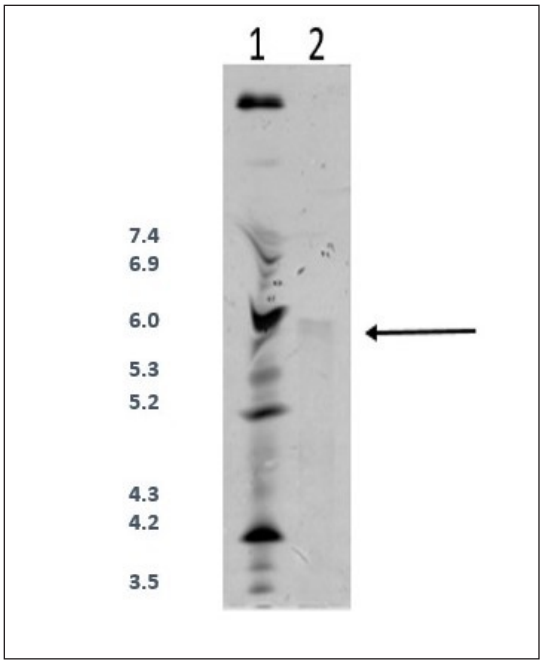

Fig. 2. Isoelectric-focusing of purified GST (10 µg). Lane 1 shows the SERVA™ IEF marker (Invitrogen); Lane 2 shows the presence of a single band of the purified protein. The estimated pI value is 6.0. The gel was stained with silver.

2003). The purified putative GST has low activity towards 1-Chloro-2,4-dinitrobenzene (CDNB) which serves as a common substrate for other GSTs classes, especially for beta class GST. The low activity with this substrate is suggesting that it is bacterial GSTs (Zablotowicz *et al*., 1995). Table 1 shows that purified GST reacted with both hydrogen peroxide and cumene hydroperoxide, thus showing selenium-independent glutathione peroxidase activity. However, the purified putative GST has no activity with trans-octenal, hepta 2,4 dienal, and trans-4-phenyl-3-butene-2-one, indicating the purified putative GST was not involved in lipid peroxidation.

Due to lacking microbial glutathione transferases databases, we were unable to further identify the purified GST as glutathione transferase through MALDI-TOF mass spectrometry analysis. However, we have obtained a total of 20 putative glutathione transferases of *Pseudomonas* sp. UW4 information from the *Pseudomonas* database, http://www.pseudomonas.com and summarized in Table 2. It revealed that among a total of 20 putative GSTs, ppUW4_00801 has close characteristics and properties to purified GST. A phylogenetic tree was constructed (Figure 3) between the total 20 putative glutathione transferases of this strain with different classes of known glutathione transferases. It is showing that ppUW4_00801 is located close to Omega class glutathione transferase. Up to date, there are no bacteria GSTs can be found under the Omega class, suggesting that it would be a new class of GST.

Table 1. Substrate specificity of purified protein from *Pseudomonas* sp. UW4. Towards selected substrates (n.d: not detected)

Substrate	Specific activity (nmoL/min/mg)
Ethacrynic acid (EA)	43.7
1-chloro-2,4-dinitrobenzene (CDNB)	9.5
Cumene hydroperoxide	6.5
Hydrogen peroxide	0.8
Trans-Octenal	n.d
Hepta 2,4 dienal	n.d.
Trans-4-phenyl-3-butene-2-one	n.d
p-nitrophenyl chloride	n.d
Sulfobromopthalein (BSP)	n.d

Table 2. All the putative glutathione transferases found in *Pseudomonas* sp. UW4

No.	Locus tag	Gene	Molecular weight (kDa)	Isoelectric point (pI)
1	PputUW4_04444	Glutathione S-Transferase	34.06	6.05
2	PputUW4_01622	Glutathione S-Transferase	26.73	5.39
3	PputUW4_01836	Glutathione S-Transferase	38.24	6.65
4	PputUW4_05417	Glutathione S-Transferase	22.48	6.52
5	PputUW4_03237	Glutathione S-Transferase	2.72	7.92
6	PputUW4_04058	Glutathione S-Transferase	23.24	5.31
7	PputUW4_04228	Glutathione S-Transferase	24.36	6.91
8	PputUW4_04088	Glutathione S-Transferase	24.51	6.8
9	PputUW4_04314	Glutathione S-Transferase	23.22	6.52
10	PputUW4_04999	Glutathione S-Transferase	24.7	5.3
11	PputUW4_05278	Glutathione S-Transferase	23.94	5.76
12	PputUW4_01937	Glutathione S-Transferase	22.37	4.56
13	PputUW4_03021	Glutathione S-Transferase	25.38	6.6
14	PputUW4_03204	Glutathione S-Transferase	25.2	6.93
15	PputUW4_01628	GST-like protein	23.11	6.3
16	PputUW4_02852	GST-like protein	23.07	5.38
17	PputUW4_00864	GST-like protein	24.33	6.68
18	PputUW4_03762	Glutathione S-Transferase (Glutathione Peroxidase)	20.61	8.19
19	PputUW4_00801	Glutathione S-Transferase (Glutathione Peroxidase)	17.6	6.1
20	PputUW4_01443	Glutathione S-Transferase (Glutathione Peroxidase)	17.6	9.27

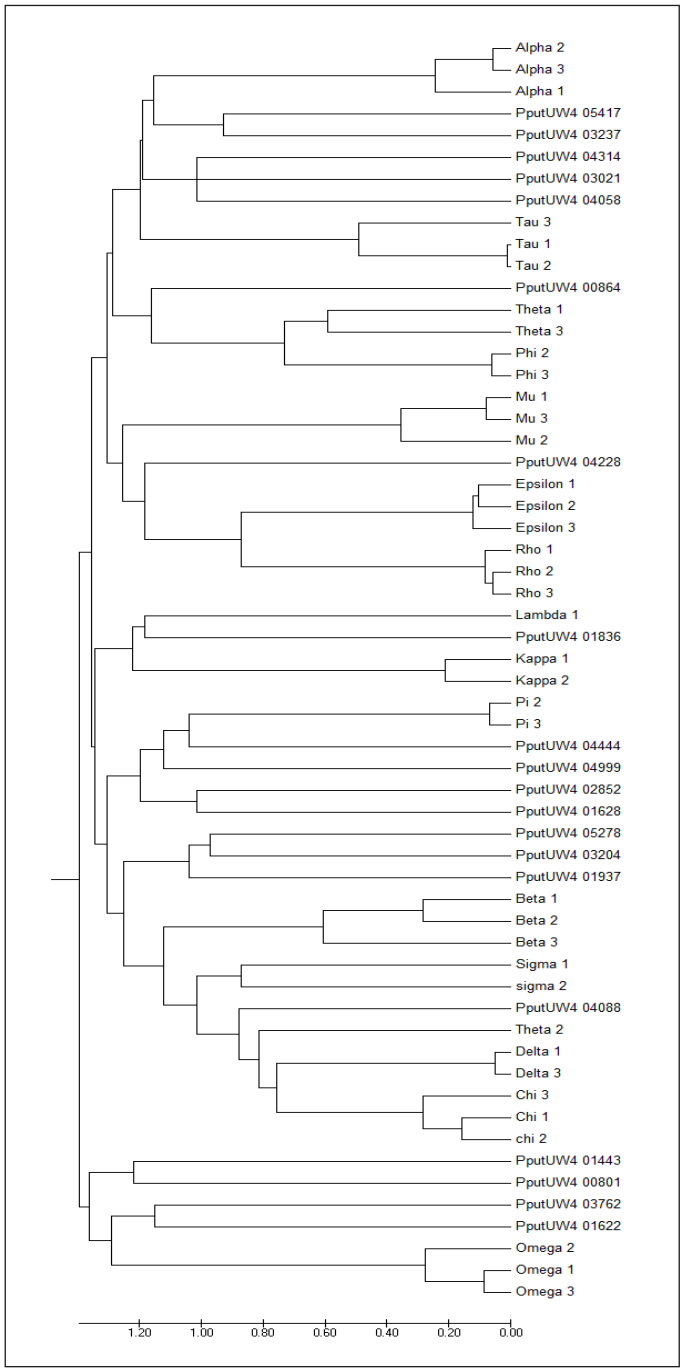

Fig. 3. Evolutionary relationships of taxa for all the glutathione found in *Pseudomonas* sp. UW4.

Beta1 (*Proteus mirabilis*; ID: WP_212637524.1), Beta2 (*Escherichia coli*; ID: WP_097734823.1), Beta3 (*Acinetobacter* sp. HR7; ID: WP_034588072.1), Alpha1 (*Homo sapiens*; ID: NP_001503.1), Alpha2 (*Mus musculus domesticus*; ID: AAA37754.1), Alpha3 (*Rattus norvegicus*; ID: NP_001100310.1), Zeta1 (*Salmonella* sp.; ID: WP_000781183.1), Zeta2 (*Arabidopsis thaliana* sp.; ID: NP_973400.1), Zeta3 (*Arabidopsis thaliana* sp.; ID: NP_178343.1), Theta1 (*Rattus norvegicus*; ID: NP_445745.1), Theta2 (*Populus trichocarpa*; ID: ADB11337.1), Theta3 (*Arabidopsis thaliana* sp.; ID: NP_198940.3), Tau1 (*Oryza sativa* Japonica Group; ID: XP_015630984.1), Tau2 (*Oryza sativa* Indica Group; ID: AAC05216.1), Tau3 (*Oryza sativa* Japonica Group; ID: NP_001390982.1), Phi2 (*Solanum commersonii*; ID: ABQ96852.1), Phi3 (*Capsicum chinense*; ID: CAI51314.2), Pi2 (*Homo sapiens*; ID: 6LLX_A), Pi3 (*Rattus norvegicus*; ID: NP_036709.1), Mu1 (*Homo sapiens*; ID: NP_000552.2), Mu2 (*Echinococcus multilocularis*; ID: CAA59739.1), Mu3 (*Mus musculus*; ID: NP_032209.1), Delta1 (*Anopheles arabiensis*; ID: XP040151601.1), Delta3 (*Anopheles gambiae;* ID: CAB03592.1), Kappa1 (*Homo sapiens;* ID: NP_057001.1), Kappa2 (*Rattus norvegicus;* ID: NP_852036.1), Omega1 (*Homo sapiens*; ID: 4YQM_A), Omega2 (*Mus musculus;* ID: NP_080895.2), Omega3 (*Sus scrofa*; ID: NP_999215.1), Chi1 (*Lyngbya* sp. PCC 8106; ID: WP_009787675.1), Chi2 (*Nodularia spumigena*; ID: WP_006196313.1), Chi3 (*Thermosynechococcus vestitus*; ID: WP_011056062.1), Rho1 (*Danio rerio*; ID: NP_001038525.1), Rho2 (*Hypophthalmichthys molitrix;* ID: ABV24478.1), Rho3 (*Cyprinus carpio;* ID: ABD67511.1), Epsilon1 (*Drosophila erecta*; ID: XP_026836720.1), Epsilon2 (*Drosophila melanogaster*; ID: NP_611323.1), Epsilon3 (*Drosophila ananassae*; ID: XP_001959218.1), Sigma1 (*Operophtera brumata*; ID: KOB75653.1), Sigma2 (*Blattella germanica*; ID: AEV23881.1), and Lambda1 (*Populus Trichocarpa*; ID: 4PQH_A)

CONCLUSION

Pseudomonas sp. UW4, a plant growth-promoting bacterial sample was found to produce a GST with a low molecular weight of 16 kDa (based on SDS-PAGE). Using isoelectric focusing, the band was revealed in a single band indicating a single isoform with pI values of 6.1. The purified putative GST can conjugate with substrates involved in peroxidation proposing it may involve in combating oxidative stress but not involved in lipid peroxidation. Purified GST of *Pseudomonas* sp. UW4 has close characteristics and properties to putative ppUW4_00801 and is located close to Omega class glutathione transferase. Up to date, most of the omega class GST can be found in humans, but not much has been detected in bacteria. More studies on bacterial GSTs are needed because of soil bacteria contribute to the resistance to environmental stresses. It plays an important role in environmental bioremediation and biodegradation. With additional engineered fusion proteins with functional versatility, they may become one of the approaches to develop applications in the biological treatment of environmental and industrial pollutants.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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