A novel deep-sea bacterial threonine dehydratase drives cysteine desulfuration and hydrogen sulfide production

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Short title: A novel cysteine desulfurase

26 **ABSTRACT**

27 Cysteine desulfuration is one of the main ways for hydrogen sulfide (H₂S) generation
 28 in cells and is usually conducted by cystathionine γ -lyase. Herein, we describe a
 29 newly discovered deep-sea bacterial threonine dehydratase (psTD), which is
 30 surprisingly discovered to drive L-cysteine desulfuration. The mechanisms of psTD
 31 catalyzing cysteine desulfuration towards H₂S production are first clarified *in vitro*
 32 and *in vivo* through a combination of genetic and biochemical methods. Furthermore,
 33 based on the solved structures of psTD and its various mutants, two or three pockets
 34 are found in the active site of psTD, and switch states between inward and outward
 35 orientation of a key amino acid R77 determine the open or close status of Pocket III
 36 for small molecule exchanges, which further facilitates cysteine desulfuration. Our
 37 results reveal the functional diversity and structural specificity of psTD towards
 38 L-cysteine desulfuration and H₂S formation. Given the broad distribution of psTD
 39 homologs in different bacteria, we speculate that some threonine dehydratases have
 40 evolved a novel function towards cysteine desulfuration, which benefits the producer
 41 to utilize cysteine as a sulfur source for better adapting external environments.

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INTRODUCTION

The important life-supporting role of hydrogen sulfide (H₂S) has been found from bacteria to plants, and finally to mammals (Wang, 2012). In bacteria, endogenous H₂S is involved in stress responses, such as oxidative stress and antibiotics, and the assembly of intracellular [Fe-S] clusters which are ubiquitous and evolutionary ancient prosthetic groups required to sustain fundamental life processes (Johnson et al, 2005; Mihara & Esaki, 2002; Mironov et al, 2017; Shatalin et al, 2011). L-cysteine is a common substrate in many bacterial species for H₂S generation, which is catalyzed by cysteine desulfurases using pyridoxal 5'-phosphate (PLP)-based chemistry (Chiku et al, 2009; Mihara & Esaki, 2002; Szabo, 2018; Wendisch, 2007). Bacterial cysteine desulfurases include cystathionine β-synthase (CBS), cystathionine γ-lyases (CSE), *O*-acetylserine sulfhydrylase (OASS), et al (Awano et al, 2005; Devi et al, 2017; Dunleavy et al, 2016). In *Escherichia coli*, five different enzymes have been identified to possess cysteine desulfurase activity, which including cystathionine β-lyase (MetC), cysteine synthase A/*O*-acetylserine sulfhydrylase A (CysK), cysteine synthase B/*O*-acetylserine sulfhydrylase B (CysM), β-cystathionase (MalY), and tryptophanase (TNaA) (Awano et al, 2005).

PLP-dependent enzymes catalyze manifold reactions of amino acid metabolism (Alexander et al, 1994). Threonine dehydratase (TD), also named threonine deaminase, belongs to the β-family of PLP-dependent enzyme catalyzing the formation of α-ketobutyrate and NH₃ from L-threonine (Schomburg & Salzmann, 1990; Simanshu et al, 2006). It also catalyzes the deamination of L-serine, L-homoserine, β-chloro-L-alanine and L-allothreonine (Schomburg & Salzmann, 1990). Two types of TD have been found in bacteria: the biosynthetic threonine dehydratase (BTD) and the catabolic threonine dehydratase (CTD) (Yu et al, 2013). BTD, encoded by the gene *ilvA*, is expressed under aerobic conditions and catalyzes the first reaction in the isoleucine biosynthesis pathway (Gallagher et al, 1998). L-isoleucine and L-valine act as an allosteric inhibitor and an activator, respectively (Gallagher et al, 1998). IlvA protein provided one of the earliest examples of feedback

inhibition (Umbarger, 1956). CTD, encoded by the gene *tdcB*, is induced anaerobically and catalyzes the first reaction in the degradation of L-threonine to propionate (Simanshu et al, 2007). Unlike IlvA, TdcB protein is insensitive to L-isoleucine and L-valine and is activated by AMP (Simanshu et al, 2006). In *E. coli*, cysteine has been shown to inhibit TD activity, resulting in transient amino acid starvation, which can be reversed by threonine (Harris, 1981). However, TD has not previously been shown to have a cysteine desulfurase activity.

In the current work, a deep-sea bacterium *Pseudomonas stutzeri* 273 was found to produce substantial amounts of H₂S in the presence of cysteine, and TD of this bacterium (psTD) was demonstrated to drive L-cysteine desulfuration. A combination of the proteomic method together with gene knockout approaches revealed that psTD drove L-cysteine desulfuration and thereby the generation of H₂S in *P. stutzeri* 273. Furthermore, structural insights of psTD mediating L-cysteine desulfuration were detailedly disclosed. Overall, this work reports a previously undocumented cysteine desulfurase activity for bacterial TD both *in vivo* and *in vitro*.

RESULTS

psTD drives L-cysteine desulfuration *in vivo*.

Originally, we observed that addition of L-cysteine promoted *P. stutzeri* 273 to generate substantial amounts of H₂S (Fig. 1A). To investigate the response of *P. stutzeri* 273 when exposed to L-cysteine, we performed proteomic analyses of *P. stutzeri* 273 incubated in LB and LB supplemented with 8 mmol/L L-cysteine. Among the significantly up-regulated proteins, four were associated with sulfur metabolism (Fig. 1B). Meanwhile, the expression of pyridoxal kinase for pyridoxal 5'-phosphate (PLP) synthesis was significantly increased (Fig. 1B). Of the four proteins, psTD is one of the proteins containing a PLP binding domain (Fig. S1), which is an obligate cofactor required for cysteine desulfuration (Majtan et al, 2018; Mihara & Esaki, 2002; Sun et al, 2009). Based on the proteomic results, we propose that psTD might involve in the cysteine metabolism of *P. stutzeri* 273.

To evaluate corresponding roles of psTD and other two cystathionine γ -lyases determining the production of H₂S in *P. stutzeri* 273, we constructed a series of deletion mutants targeting the genes encoding psTD (ΔTD), cystathionine γ -lyase 1 ($\Delta CSE1$), cystathionine γ -lyase 2 ($\Delta CSE2$), or different combinations. Thereafter, cell extracts from wild-type *P. stutzeri* 273 and the corresponding mutants were analyzed using in-gel activity assays for detecting the potential H₂S-producing enzymes. Clearly, wild-type *P. stutzeri* 273 could degrade L-cysteine to produce H₂S, as could the mutants $\Delta CSE1$, $\Delta CSE2$, and $\Delta CSE1\Delta CSE2$ (Fig. 1C). However, the ability to generate H₂S was compromised in ΔTD (Fig. 1C), which showed lower levels of H₂S production than wild-type *P. stutzeri* 273 (PS273), $\Delta CSE1$, $\Delta CSE2$, and $\Delta CSE1\Delta CSE2$ (Fig. 1D). Complementation ΔTD with the wild-type psTD gene ($\Delta TD/cTD$) restored H₂S production (Fig. 1C). Thus, we conclude psTD plays a key role in catalyzing L-cysteine desulfuration and H₂S formation in *P. stutzeri* 273 *in vivo*.

psTD drives L-cysteine desulfuration *in vitro*.

To determine whether psTD was capable of catalyzing H₂S generation from L-cysteine *in vitro*, it was over-expressed and purified from the *E. coli* BL21 cell line. Recombinant psTD showed a strong absorbance at 412 nm, a typical symbol of covalently bound PLP (Fig. 2A), and it is consistent with the presence of a PLP binding domain in psTD (Fig. S1). psTD generated H₂S from L-cysteine in the in-gel activity assay (Fig. 2A), consistent with the *in vivo* assay (Fig. 1C). Moreover, we identified L-serine as one of the downstream products (Fig. 2B) based on a previous report (Chiku et al, 2009). Pyruvate, a downstream product of L-serine, was also detected by spectrophotometry (Fig. 2C). In addition, psTD had a more substantial catalytic velocity when compared to other CSEs according to the intrinsic kinetics of the reaction (Table S2). Together, we propose that psTD catalyzes the following reaction: L-cysteine + H₂O $\rightarrow$ L-serine + H₂S $\rightarrow$ pyruvate + NH₃ + H₂S (Fig. 2D).

Structural basis of psTD catalyzing L-cysteine desulfuration.

To better understand the catalytic mechanism of psTD-driven L-cysteine desulfuration, the crystal structures of psTD wild type and corresponding mutant, and complex

131 bound with PLP were determined (Table 1). The crystal structures of psTD were
 132 solved around 1.5~2Å in two conditions, C6 (PDB 7DAP) and E11 (with high
 133 concentration NH_4^+ , PDB 7DAQ). The structure of psTD is an asymmetric
 134 homodimer possessing two similar subunits (superposition with RMSD=0.20Å) (Fig.
 135 S2A). Each subunit is highly conserved to the structure of 1TDJ (TD from *E. coli*,
 136 superposition with RMSD=1.67Å), although the identity is only 31% between two
 137 sequences (Fig. S2B).

138 As PLP is a co-enzyme of TD and is essential for TD to perform catalytic activity,
 139 thus co-crystal structure of complex containing psTD and PLP was solved and
 140 analyzed (PDB 7D8Y). The structural results show that PLP binds to the amino acid
 141 K51 with both covalent and non-covalent bonds and occupies one pocket of active
 142 site from several resolved crystals (Fig. 3A). Consistently, the sequence alignment of
 143 TD protein families indicates that the key residue of active site is K51 (Fig. S3A). It is
 144 noting that PLP may be missing in the site when high concentration NH_4^+ is present in
 145 the crystallization buffer since NH_4^+ may occupy one or two pockets around K51 in
 146 the active site (Fig. 3B). Moreover, the sidechain of the amino acid R77 interacts with
 147 PLP in C6 crystal structure while it is orientating to protein surface in high NH_4^+
 148 crystal structure since the active site is occupied by NH_4^+ (Fig. 3C). Therefore, NH_4^+
 149 may prevent the sidechain of R77 from orientating inside of protein as well as binding
 150 with PLP, which is consistent with the proposal that NH_4^+ (as a catalytic product) may
 151 reduce substrate concentration and slow down the reaction as negative feedback (Figs.
 152 2D and 3B). The reduced activity of psTD caused by NH_4^+ further confirms this view
 153 (Fig. 3D). Interestingly, the conformation change and alternative conformation of R77
 154 sidechain observed in chain A and chain B imply the dynamics and specific function
 155 of R77 (Fig. 3C and Fig. S4A). To explore the potential function of R77, the structure
 156 of psTD with mutation of R77E (R is mutated to E; PDB 7DAR) was also solved for
 157 comparison. The results show that there are alternative sidechains for E77 in chain A
 158 and alternative mainchain for amino acid G78 in chain B of 7DAR, where the
 159 sidechain of amino acid E77 is extending to protein surface (Fig. 3E).

160 The inside active sites in different psTD structures were further compared by the
161 map of electrostatic potential (MEP). PLP binds deep inside the psTD structure with
162 positive charged pockets around catalytic key residue K51 (Fig. 4A). Besides, the
163 active site of psTD is unique in sharp and could be switchable at the gate position near
164 R77 in some structure states (Fig. 4B). In the structure 7DAQ, there are an amino acid
165 residue bound by K51 and two NH_4^+ ions instead of PLP in chain A, while PLP with
166 weak density is present in chain B with non-covalent binding to K51, together with
167 one NH_4^+ ion in another pocket (Figs. 3B and 4B). In the structure 7DAR, the E77
168 with negative charged sidechain may block the gate of the active site by negative
169 potential gap at the psTD surface (Fig. 4C). Consistently, psTD with mutation R77E
170 significantly reduces the catalytic activity to form H_2S (Fig. 3D).

171 Furthermore, there are intensive hydrogen-bond interactions between phosphate
172 group of PLP and amino acids G177-G181 (GLGSG), A277, S302 as well as the salt
173 bridge interaction between phosphate group of PLP and the specific residue R77,
174 therefore these residues may stabilize the PLP binding (Fig. 5). Notably, the sequence
175 motifs around amino acids F50-K51, A75-H80 and G177-G181 are conserved through
176 whole TD family (Fig. 5E), indicating the threonine dehydratase function of psTD is
177 conserved and ubiquitously exists in different microorganisms. While the unique
178 cysteine desulfuration may result from specific structure and motifs associated with
179 the diversity positions, such as R77 and the relevant residues near active site.

180 **Two or three pockets in the active site determine the catalytic function of psTD.**

181 Notably, MEP comparison of active sites between structures 7D8Y (psTD-PLP
182 complex) and 1TDJ (TD homolog from *E. coli*) shows that the active site for 7D8Y is
183 close to U shaped channel which could be divided into two or three pockets (Fig. 6
184 and Fig. 7). Pocket I (PI) is occupied by PLP and Pocket II (PII) is on another side of
185 K51 (Figs. 7A and 7B). Pocket III (PIII), the access channel, opens at the surface of
186 7D8Y around R77 and only presents with R77 outward orientation (Figs. 6, 7C, and
187 7D). The residues interacting with PLP (as PI of active site) are highly conserved at
188 the structural level except flexibility of R77 and G181, although the sequence motifs

are relatively similar, together with some diversity positions at T76, R77, L178, S180, G181, A277, S302, et al (Fig. S5B). The structural conservation is consistent with the phylogenetic classification and sequence motifs of TD family (Figs. S2E, S2F, S3B, S7). Interestingly, there is only one pocket to hold PLP at the active sites from 1TDJ, 1P5J, 6VJU (1P5J: Ser dehydratase, 6VJU: Cys synthase), which is directly opened to protein surface (corresponding to PIII) and mostly conserved with PI of 7D8Y at the structural level (Fig. 6). The small molecules of reaction products are favorable to bind in PII, which may result in multiple functions including cysteine desulfuration based on its structure and sequence specificity. Besides the structure difference with pockets, there is also some sequence diversity at L151, C233 for PII and PIII, respectively (Fig. S5).

As mentioned above, the R77 has alternative sidechain conformations in 7D8Y: one is interacting with PLP (inward state with PI and PII), and another is orienting to protein surface (outward state with additional PIII) (Fig. 7E). Active site could expand more space between pockets and protein surface, so that small molecules could access the gate of the active site and reach PLP to trigger the further reactions for R77 outward conformation (Fig. 7). The psTD with mutation of R77A could remain comparable enzyme activity since the gate of active site may keep opened like 1TDJ (with small sidechain of Ala at corresponding position), while psTD with the mutation R77E loses its function since its negative charge may block the small molecules to access the active site (Figs. 3D, 6E, S5A). Therefore, residues near R77 may play a role as gate-keepers controlling the entry to and from the active site, which has shown some extended flexibility of the sidechain of R77 and the mainchain near R77 (Pravda et al, 2014), while the function and mechanism for dynamics of PIII may need further study.

DISCUSSION

In the present study, detailed biochemical and structural mechanisms of psTD driving L-cysteine desulfuration are first disclosed. The initial clue of psTD mediating

217 L-cysteine desulfuration comes from the proteomic and genetic results (Fig. 1), which
 218 propose psTD as a new candidate for metabolizing L-cysteine in the deep-sea
 219 bacterium *P. stutzeri* 273 (Fig. 1). TD is principally known for catalyzing L-threonine
 220 or L-serine deamination in microorganisms (Ernst & Downs, 2018; Favrot et al, 2018;
 221 Lambrecht et al, 2013). In this study, threonine dehydrase was identified as a new
 222 member of cysteine desulfurases, and the alternative activities of enzymes play an
 223 important role in the diversification of enzymes (O'Brien & Herschlag, 1999). We
 224 speculate cysteine desulfuration catalyzed by psTD might contribute to the
 225 organic/inorganic sulfur of the deep-sea sediment. Overall, the discovery of cysteine
 226 desulfurase activity of psTD introduced a new metabolic pathway for the H₂S
 227 enzymatic production (Wang, 2012).

228 With biochemical method, the basic process of cysteine desulfuration mediated
 229 by psTD is clear (Figs. 2B-2D), however, the deep catalyzing mechanisms are still
 230 obscure. With this, we further investigated the underlying mechanisms by structural
 231 methods. Based on the structure of psTD, its mutant and complex with PLP, we
 232 clarify that the active site with positive charge can be recognized as two or three
 233 major pockets for PLP (PI) and substrates (PII) binding or the small molecules
 234 exchange (PIII), which are essential in cysteine desulfuration mediated by psTD. PII
 235 and PIII may play a key role in helping determine enzyme specific substrates
 236 (cysteine and serine) as well as specific functions (like cysteine desulfuration, serine
 237 dehydratase) (Figs. 6 and 7). NH₄⁺ may occupy one or two pockets and block the PLP
 238 binding as well as the R77 in-ward conformation (Fig. 4 and Fig. S4). Given that
 239 NH₄⁺ is a possible catalytical product of the cysteine desulfuration catalyzed by psTD
 240 (Fig. 2D), this phenomenon might be a feedback inhibition of the enzymatic reaction
 241 (Figs. 3, 4, and 7E), which is confirmed by the in-gel activity assay (Fig. 3D). On the
 242 other hand, R77 with inward orientation can stabilize PLP binding through salt bridge
 243 interaction in PI, while R77 with outward orientation may open an access channel
 244 (PIII) of active site (Fig. 7). The mutation analysis shows R77E significantly changes
 245 the charge of gate residue as well as the flexibility of main-chain around residues

246 76-78, which leads to block small molecular traffics (Fig. 4). The MEP of active site
 247 and dynamics and flexibility of R77 may play a key role in enzyme activity, which is
 248 similar to the roles of R62 from hCGL and its structural dynamics described in
 249 previous report (Yan et al, 2017). R77, as the gate-keeper, is a potential switch to
 250 control the small molecules exchange at the gate and in the active site as well as the
 251 reaction type and process at active site (Fig. 7E). Overall, the above results shed light
 252 on mechanisms of psTD catalyzing cysteine to form H₂S, which consists well with the
 253 results disclosed by genetic, proteomic and biochemical results in this study.

254 Altogether, we first show that psTD mediates cysteine desulfuration both *in vivo*
 255 and *in vitro*. Given the broad distribution of psTD homologs in different bacteria, we
 256 speculate that some threonine dehydratases have evolved a novel function towards
 257 cysteine desulfuration, which benefits the producer to utilize cysteine as a sulfur
 258 source for better adapting external environments.

259 MATERIALS AND METHODS

260 Strains, media, and chemicals.

261 *Pseudomonas stutzeri* 273 was isolated from the sediment samples collected by RV
 262 KEXUE in the East China Sea in the year of 2014 (Wu et al, 2016). *P. stutzeri* 273 and
 263 its mutants were incubated in LB broth (10 g/L peptone, 10 g/L NaCl, 5 g/L yeast
 264 extract, pH 7.0) under vigorous agitation at the speed of 150 rpm at 28 °C.
 265 *Escherichia coli* DH5α was used as the host for plasmid construction. *E. coli* S17-1
 266 was used as a vector donor in conjugation. *E. coli* BL21 was used for recombinant
 267 protein overproduction. *E. coli* DH5α, *E. coli* SY327, *E. coli* S17-1, and *E. coli* BL21
 268 were grown in LB medium at 37 °C with shaking speed of 150 rpm. When necessary,
 269 antibiotics were used at the following final concentrations: 25 µg/mL chloramphenicol
 270 (Cm), 25 µg/mL gentamicin (Gm), 100 µg/mL ampicillin (Amp), and 100 µg/mL
 271 kanamycin (Kan) (Zhang et al, 2020).

272 H₂S production assay.

273 To detect H₂S production of *P. stutzeri* 273, bacterial cells were transferred into 5 ml
274 of LB with the addition of L-cysteine in glass tubes (18*150 mm). Paper strips with
275 lead acetate were affixed at the top of the tubes with rubber stoppers. After shaking
276 incubation for 12 h, the paper strips were photographed to detect the presence of black
277 lead sulfide precipitates, which is correlated to the production of H₂S. The estimation
278 was done by visually matching the darkness of the paper strips (Xia et al, 2017).

279 **Proteomic analysis.**

280 *P. stutzeri* 273 cells were incubated in LB and LB with 8 mmol/L L-cysteine until
281 OD₆₀₀ was 0.8. Then proteins were extracted, separated and identified using liquid
282 chromatography- tandem mass spectrometry (LC-ESI-MS/MS) analysis. The
283 bioinformatic analyses of protein annotation, functional classification, functional
284 enrichment and cluster analyses were performed. The heat map of differently
285 expressed proteins (1.5-fold change cutoff and *P* value less than 0.05) was made by
286 the software Heml 1.0.3.3.

287 **Bioinformatics analysis.**

288 *P. stutzeri* 273 was collected in China General Microbiological Culture Collection
289 Center under collection number CGMCC 7.265. The complete genome sequence of *P.*
290 *stutzeri* 273 has been deposited at GenBank under the accession number CP015641
291 (Wu et al, 2017). The gene sequences of *threonine dehydratase* (*psTD*, accession
292 number PS273GM_RS04065), *cystathionine γ-lyase1* (*CSE1*, accession number
293 PS273GM_RS00775) and *cystathionine γ-lyase2* (*CSE2*, accession number
294 PS273GM_RS06855) were obtained from GenBank. The consensus phylogenetic tree
295 of *psTD* in *P. stutzeri* 273 with other related proteins obtained from GenBank was
296 constructed by the maximum likelihood method with MEGA 7.0 (Kumar et al, 2016).
297 Weblogo of amino acid residues according to the multiple sequence alignment with
298 *psTD* and other proteins in TD family (MSA-TD) was created in Weblogo
299 (<http://weblogo.berkeley.edu/logo.cgi>). The MSA-TD was mapped to the *psTD*

300 structure using ConSurf Server (<https://consurf.tau.ac.il/>) (Ashkenazy et al, 2010).
301 The proteins used in MSA-TD are shown in Supplementary, Fig. S3B.

302 **Construction of deletion mutants and complementation strains in *P. stutzeri* 273.**

303 Gene knockout in *P. stutzeri* 273 was made following the conjugation method
304 described previously (Wu et al, 2017; Zheng et al, 2020). Fragments for mutant
305 construction were amplified from the chromosome of *P. stutzeri* 273 by primers
306 shown in Supplementary Table S1. Purified homologous fragments upstream and
307 downstream of the target region were digested and ligated into the suicide vector
308 pEX18Gm containing an *oriT* for conjugation. The constructed vector was transferred
309 into *E. coli* SY327 and then *E. coli* S17-1 in turn. Using *E. coli* S17-1 as a donor
310 strain, the constructed vector was transferred into *P. stutzeri* 273 by intergeneric
311 conjugation at 28 °C for 48 h. After mating, cells were plated on LB agar plate with
312 Cm and Gm to screen for single-event positive recombinant strains. The individual
313 colony was used for second crossover. Sucrose counter-selection produced mutants
314 without the pEX18Gm region. All double-recombination mutant candidates were
315 verified by PCR amplification and sequencing. The primers used for validating
316 complete removal of the target region from the host genome were shown in
317 Supplementary Table S1.

318 The plasmid pUCP18 was used to construct complementary strains. The gene of
319 *psTD* together with its native promoter was amplified from the wild-type *P. stutzeri*
320 273 by primers listed in Supplementary Table S1. The purified PCR product was
321 inserted into *HindIII/BamHI* site of pUCP18 to produce pUCP18-*TD*. Then
322 pUCP18-*TD* was transferred into mutant strain ΔTD . The final complementary strain
323 $\Delta TD/cTD$ was verified by PCR amplification and sequencing.

324 **In-gel detection of H₂S producing enzyme.**

325 The enzymes degrading L-cysteine and forming H₂S were detected using an in-gel
326 activity assay with bismuth staining (Basic et al, 2017; Yoshida et al, 2010). Briefly,
327 the cell lysates obtained by sonication were applied to Native-PAGE gel (12%). After

electrophoresis, the gel was incubated in 100 mmol/L triethanolamine-HCl pH 7.6, 10 μ mol/L pyridoxal 5-phosphate monohydrate, 1.0 mmol/L bismuth trichloride, 10 mmol/L EDTA and 20 mmol/L L-cysteine at 37 °C for 30 min or 3 h. H₂S formed during the enzymatic reaction precipitated as insoluble bismuth sulfide, and H₂S producing enzymes appeared as brown to black bands in the gels.

Expression and purification of psTD or its mutants.

The gene encoding psTD or its mutant was cloned into a pET-28a expression vector incorporating an N-terminal His tag fusion. The vector pET-28a containing psTD encoding gene was used as a template for construction of different mutants of psTD. The mutation in the 77th amino acid (R77) of psTD was introduced by site-directed mutagenesis using KOD -Plus- Mutagenesis Kit (TOYOBO, Japan) to express psTD R77A (R is replaced with A), R77E (R is replaced with E), and R77K (R is replaced with K), respectively. The primers used for site-directed mutagenesis were shown in Supplementary Table S1. To express psTD or its mutants, plasmid containing different target gene was transformed into *E. coli* BL21 cells (Zheng et al, 2020). An overnight culture of *E. coli* BL21 cells containing different expression vector was inoculated into 1 L LB broth. Cultures were grown for 3 h at 37 °C with aeration (150 rpm) until an OD₆₀₀ of 0.8 was reached. Then the expression was induced by 0.1 mmol/L IPTG at 16 °C for 12 h. Thereafter, cells were collected by centrifugation (8,000 $\times$ g, 20 min), resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and subjected to sonication. Following lysis, the extract was centrifuged, filtered, and injected into a HisTrap high-performance (HP) column (5 mL) (GE Healthcare, America). The proteins were eluted with increasing concentrations of imidazole buffer (50 mM Tris, 150 mM NaCl, and 50-250 mM imidazole). After overnight dialysis to Tris buffer (10 mM Tris, 150 mM NaCl, 10% glycerol, pH 8.0), the protein solution was applied to a HiTrapTM Q HP column (GE Healthcare) and eluted with linear gradient of 0.15-2.0 M of NaCl in 10 mM Tris (pH 8.0). After overnight dialysis to Tris buffer (10 mM Tris, 150 mM NaCl, pH 8.0), active fractions were collected and concentrated by ultrafiltration (MWCO 10 kDa, Millipore), and loaded onto a HiloadTM 16/600

357 superdexTM 200 column (GE Healthcare, USA) pre-equilibrated with 10 mM Tris
358 (pH 8.0) containing 150 mM NaCl. Bound proteins were eluted with the equivalent
359 buffer at a flow rate of 1 ml/min. The purity of fractions collected was determined by
360 SDS-PAGE. Protein aliquots were quick-frozen in liquid nitrogen and stored in -80 °C
361 till future use.

362 **Liquid chromatography-mass spectrometry (LC-MS) analysis.**

363 To find out the products from L-cysteine catalyzed by psTD, the mixture of 20
364 mmol/L L-cysteine and 0.1 mg/mL psTD in Tris buffer (50 g/L Tris, 150 g/L NaCl,
365 pH 7.5) was incubated at 37 °C for 2 h. After protein precipitation by trichloroacetic
366 acid, 1.75 mL borate buffer (1 mol/L, pH 9.0), 750 µL methanol, 1 mL enzymatic
367 mixture, and 30 µL of diethyl ethoxymethylenemalonate (DEEMM) were mixed in a
368 screw-cap test tube, treated in ultrasound bath over 30 min, and then heated at 70 °C
369 for 2 h allow complete degradation of excess DEEMM and reagent byproducts
370 (Gomez-Alonso et al, 2007). After derivatization, the reaction products were analyzed
371 by liquid chromatography (Agilent Technologies)-mass spectrometry (BRUKER,
372 maxis plus) (LC-MS). Pyruvate concentration in the enzymatic mixture was measured
373 using 2,4-dinitrophenylhydrazine by monitoring the absorbance at 515 nm in relation
374 to a standard curve (Anthon & Barrett, 2003).

375 **Crystallization and structural determination.**

376 Purified psTD or corresponding mutant was mixed with PLP (2 mmol/L) to a final
377 concentration at 10.8 mg/mL (psTD or mutants) in the buffer containing 20 mmol/L
378 Tris-HCl, 150 mmol/L NaCl, 1 mmol/L β-mercaptoethanol, pH 8.0. psTD, its
379 corresponding mutant or psTD-PLP/psTD mutant-PLP complexes were screened for
380 crystallization conditions using sitting-drop vapor diffusion at 6, 12 and 18 µg/mL.
381 Crystals were formed in 1.8 µmol/L ammonium sulfate, 0.1 mol/L Bis-Tris (pH 6.5), 2%
382 (v/v) PEG 550 (E11 condition), or 0.1 mol/L NaAc·3H₂O (pH 4.6), 3.5 mol/L
383 HCOONa (C6 condition) within a week. Crystals were harvested and soaked in well
384 solution containing 25% glycerol before flash frozen in liquid nitrogen. Crystals data

385 were collected at 100 K at the beamline BL17U1 and BL19U1 at the Shanghai
386 Synchrotron Radiation Facility (SSRF, China). The homolog model of TD was used
387 for molecular replace with PHENIX, and the structure model was manually modified
388 and refined by PHENIX through iterative cycles. The final refinement statistics are
389 given in Table 1. All Figures were created with PyMOL (<http://www.pymol.org/>).

390 **Statistical analysis.**

391 All experiments were performed in triplicate and the data were expressed as mean $\pm$
392 standard deviation. The statistical analyses were performed with one-way analysis of
393 variance (ANOVA). A multiple comparison Tukey test was used to evaluate if
394 significant differences among treatments existed.

395 **Data availability.**

396 All proteomics related data have been deposited to the ProteomeXchangeConsortium
397 via the PRIDE partner repository (dataset identifier PXD011469). The structures of
398 psTD (C6 condition, native structure with PLP bound), psTD (E11 condition), psTD
399 (psTD-PLP co-crystal complex with C6 condition), and psTD mutant (R77E mutation
400 with C6 condition) have been deposited in the Protein Data Bank (PDB) under the
401 accession codes 7DAP, 7DAQ, 7D8Y, and 7DAR, respectively.

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411 **Author contributions**

412 NM and CS conceived and designed the experiments. NM performed majority of the
413 experiments and analyzed the data. NM, YS, and WZ purified proteins. YS, WZ
414 design the crystallization experiments, grew crystals, collected data, solved the
415 structures and carried out the structure analysis. NM, WZ and CS prepared the
416 Figures and wrote the paper with all the inputs of all authors.

417 **Competing interests**

418 The authors declare no competing interests.

419 **References**

- 420 Alexander FW, Sandmeier E, Mehta PK, Christen P (1994) Evolutionary relationships among
421 pyridoxal-5'-phosphate-dependent enzymes. Regio-specific alpha, beta and gamma families. *Eur J*
422 *Biochem* **219**: 953-960
- 423 Anthon GE, Barrett DM (2003) Modified method for the determination of pyruvic acid with
424 dinitrophenylhydrazine. In the assessment of onion pungency. *J Sci Food Agr* **83**: 1210-1213
- 425 Ashkenazy H, Erez E, Martz E, Pupko T, Ben-Tal N (2010) ConSurf 2010: calculating evolutionary
426 conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res* **38**:
427 W529-W533
- 428 Awano N, Wada M, Mori H, Nakamori S, Takagi H (2005) Identification and functional analysis of
429 Escherichia coli cysteine desulfhydrases. *Appl Environ Microb* **71**: 4149-4152
- 430 Basic A, Blomqvist M, Dahlén G, Svensäter G (2017) The proteins of *Fusobacterium* spp. involved in
431 hydrogen sulfide production from L-cysteine. *BMC Microbiol* **17**: 61
- 432 Chiku T, Padovani D, Zhu W, Singh S, Vitvitsky V, Banerjee R (2009) H₂S biogenesis by human
433 cystathionine γ -lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is
434 responsive to the grade of hyperhomocysteinemia. *J Biol Chem* **284**: 11601-11612
- 435 Devi S, Rehman SAA, Tarique KF, Gourinath S (2017) Structural characterization and functional
436 analysis of cystathionine beta-synthase: an enzyme involved in the reverse transsulfuration pathway of
437 *Bacillus anthracis*. *Febs J* **284**: 3862-3880
- 438 Dunleavy R, Lu L, Kiely CJ, McIntosh S, Berger BW (2016) Single-enzyme biomineralization of
439 cadmium sulfide nanocrystals with controlled optical properties. *Proc Natl Acad Sci* **113**: 5275
- 440 Ernst DC, Downs DM (2018) Mmf1p couples amino acid metabolism to mitochondrial DNA
441 maintenance in *Saccharomyces cerevisiae*. *Mbio* **9**: e00084-00018
- 442 Favrot L, Amorim Franco TM, Blanchard JS (2018) Biochemical characterization of the
443 *Mycobacterium smegmatis* threonine deaminase. *Biochemistry* **57**: 6003-6012
- 444 Gallagher DT, Gilliland GL, Xiao GY, Zondlo J, Fisher KE, Chinchilla D, Eisenstein E (1998)
445 Structure and control of pyridoxal phosphate dependent allosteric threonine deaminase. *Structure* **6**:
446 465-475
- 447 Gomez-Alonso S, Hermosin-Gutierrez I, Garcia-Romero E (2007) Simultaneous HPLC analysis of
448 biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples.

449 *J Agric Food Chem* **55**: 608-613

450 Harris CL (1981) Cysteine and growth inhibition of *Escherichia coli*: threonine deaminase as the target

451 enzyme. *J Bacteriol* **145**: 1031-1035

452 Johnson DC, Dean DR, Smith AD, Johnson MK (2005) Structure, function, and formation of biological

453 iron-sulfur clusters. *Annu Rev Biochem* **74**: 247-281

454 Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0

455 for bigger datasets. *Mol Biol Evol* **33**: 1870-1874

456 Lambrecht JA, Schmitz GE, Downs DM (2013) RidA proteins prevent metabolic damage inflicted by

457 PLP-dependent dehydratases in all domains of life. *Mbio* **4**: 8

458 Majtan T, Krijt J, Sokolova J, Krizkova M, Ralat MA, Kent J, Gregory JF, Kozich V, Kraus JP (2018)

459 Biogenesis of hydrogen sulfide and thioethers by cystathionine beta-synthase. *Antioxid Redox Sign* **28**:

460 311-323

461 Mihara H, Esaki N (2002) Bacterial cysteine desulfurases: their function and mechanisms. *Appl*

462 *Microbiol Biot* **60**: 12-23

463 Mironov A, Seregina T, Nagornyykh M, Luhachack LG, Korolkova N, Lopes LE, Kotova V, Zavilgelsky

464 G, Shakulov R, Shatalin K, Nudler E (2017) Mechanism of H₂S-mediated protection against oxidative

465 stress in *Escherichia coli*. *P Natl Acad Sci USA* **114**: 6022-6027

466 O'Brien PJ, Herschlag D (1999) Catalytic promiscuity and the evolution of new enzymatic activities.

467 *Chem Biol* **6**: R91-R105

468 Pravda L, Berka K, Svobodová Vařeková R, Sehnal D, Banáš P, Laskowski RA, Koča J, Otyepka M

469 (2014) Anatomy of enzyme channels. *BMC Bioinformatics* **15**: 379

470 Schomburg D, Salzmann M (1990) *Threonine dehydratase*: Springer Berlin Heidelberg.

471 Shatalin K, Shatalina E, Mironov A, Nudler E (2011) H₂S: a universal defense against antibiotics in

472 bacteria. *Science* **334**: 986-990

473 Simanshu DK, Chittori S, Savithri HS, Murthy MRN (2007) Structure and function of enzymes

474 involved in the anaerobic degradation of L-threonine to propionate. *J Biosciences* **32**: 1195-1206

475 Simanshu DK, Savithri HS, Murthy MRN (2006) Crystal structures of *Salmonella typhimurium*

476 biodegradative threonine deaminase and its complex with CMP provide structural insights into

477 ligand-induced oligomerization and enzyme activation. *J Biol Chem* **281**: 39630-39641

478 Sun QX, Collins R, Huang SF, Holmberg-Schiavone L, Anand GS, Tan CH, Van-Den-Berg S, Deng

479 LW, Moore PK, Karlberg T, Sivaraman J (2009) Structural basis for the inhibition mechanism of

480 human cystathionine gamma-lyase, an enzyme responsible for the production of H₂S. *J Biol Chem* **284**:

481 3076-3085

482 Szabo C (2018) A timeline of hydrogen sulfide (H₂S) research: From environmental toxin to biological

483 mediator. *Biochem Pharmacol* **149**: 5-19

484 Umbarger HE (1956) Evidence for a negative-feedback mechanism In the biosynthesis of isoleucine.

485 *Science* **123**: 848-848

486 Wang R (2012) Physiological implications of hydrogen sulfide: a whiff exploration that blossomed.

487 *Physiol Rev* **92**: 791-896

488 Wendisch VF (2007) *Amino acid biosynthesis pathways, regulation and metabolic engineering*:

489 Springer Berlin Heidelberg.

490 Wu S, Liu G, Jin W, Xiu P, Sun C (2016) Antibiofilm and anti-infection of a marine bacterial

491 exopolysaccharide against *Pseudomonas aeruginosa*. *Front Microbiol* **7**: 102

492 Wu S, Zheng R, Sha Z, Sun C (2017) Genome Sequence of *Pseudomonas stutzeri* 273 and

493 identification of the exopolysaccharide EPS273 biosynthesis locus. *Mar Drugs* **15**: 218
494 Xia Y, Lü C, Hou N, Xin Y, Liu J, Liu H, Xun L (2017) Sulfide production and oxidation by
495 heterotrophic bacteria under aerobic conditions. *Isme J* **11**: 2754
496 Yan WP, Stone E, Zhang YJ (2017) Structural snapshots of an engineered cystathionine-gamma-lyase
497 reveal the critical role of electrostatic interactions in the active site. *Biochemistry-Us* **56**: 876-885
498 Yoshida Y, Ito S, Tamura H, Kunimatsu K (2010) Use of a novel assay to evaluate enzymes that
499 produce hydrogen sulfide in *Fusobacterium nucleatum*. *J Microbiol Methods* **80**: 313
500 Yu XF, Li Y, Wang XY (2013) Molecular evolution of threonine dehydratase in bacteria. *Plos One* **8**: 7
501 Zhang J, Liu R, Xi SC, Cai RN, Zhang X, Sun CM (2020) A novel bacterial thiosulfate oxidation
502 pathway provides a new clue about the formation of zero-valent sulfur in deep sea. *Isme J* **14**:
503 2261-2274
504 Zheng RK, Wu SM, Sun CM (2020) MerF is a novel regulator of deep-sea *Pseudomonas stutzeri*
505 flagellum biogenesis and motility. *Environ Microbiol*
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510 **Table 1** Diffraction data and refinement statistics of psTD, psTD/PLP and psTD (R77E).

Crystals	psTD (7DAQ)	(E11)	psTD (7DAP)	(C6)	psTD (7D8Y)	(PLP)	psTD (7DAR)	(R77E)
Data collection								
Resolution range	34.41-1.451 (1.503-1.451)		27.707-1.662 (1.721-1.662)		26.85- 1.659 (1.718-1.659)		35.579-1.698 (1.741-1.698)	
Space group	P 62		P 62		P 62		P 62	
Unit cell a, b, c (Å)	110.976	110.976	110.829	110.829	110.975	110.975	110.801	110.801
α, β, γ (°)	107.413	90 90 120	106.895	90 90 120	106.655	90 90 120	106.738	90 90 120
Total reflections	1054052	(28804)	1720349	(139484)	1797197	(181118)	1623851	(135029)
Unique reflections	110754	(13117)	87472	(4862)	87968	(8766)	80107	(4658)
Multiplicity	9.5	(5.4)	19.7	(16.0)	20.3	(17.7)	20.3	(16.9)
Completeness (%)	99.86	(98.86)	92.76	(55.74)	99.79	(99.85)	94.35	(58.33)
Mean I/sigma(I)	12.43	(0.14)	12.59	(0.79)	15.89	(2.77)	11.86	(1.25)
Wilson B-factor	17.80		23.88		19.59		14.54	
R-merge	0.12	(8.798)	0.1768	(3.885)	0.1459	(0.8539)	0.1946	(2.143)
R-meas	0.1268	(9.73)	0.1815	(4.012)	0.1495	(0.8754)	0.1996	(2.21)
R-pim	0.0403	(4.046)	0.041	(1.002)	0.03272	(0.1922)	0.04399	(0.534)
CC1/2	0.999	(0.0437)	0.998	(0.3)	0.989	(0.914)	0.973	(0.513)
CC*	1	(0.289)	1	(0.679)	0.997	(0.977)	0.993	(0.824)
Refinement								
Reflections used in refinement	131993	(13076)	81161	(4862)	87806	(8766)	75643	(4659)
Reflections used for R-free	1992	(199)	1987	(119)	1993	(198)	1972	(118)
R-work	0.1701	(0.2568)	0.1831	(0.2936)	0.1611	(0.2065)	0.1669	(0.2548)
R-free	0.1785	(0.2603)	0.2032	(0.2962)	0.1827	(0.2242)	0.1902	(0.2656)
CC(work)	0.471	(-0.013)	0.961	(0.701)	0.964	(0.902)	0.941	(0.794)
CC(free)	0.474	(0.094)	0.954	(0.712)	0.953	(0.892)	0.935	(0.739)
Number of non-hydrogen atoms	5305		5179		5405		5355	
macromolecules	4756		4766		4784		4772	
Protein residues	636		638		637		637	
Deviation from identity								
RMS(bonds)	0.005		0.008		0.007		0.006	
RMS(angles)	0.80		0.83		0.89		0.82	
Average B-factor	21.57		27.98		23.24		17.92	
Ramachandran plot								
Ramachandran favored (%)	98.89		98.74		98.74		99.05	
Ramachandran allowed (%)	1.11		1.26		1.26		0.95	
Ramachandran outliers (%)	0.00		0.00		0.00		0.00	
Rotamer outliers (%)	0.00		0.00		0.00		0.00	

Figures

Fig. 1 Key roles of psTD in driving intracellular H₂S generation through degrading L-cysteine in *P. stutzeri* 273. (A) H₂S production of *P. stutzeri* 273 challenged with different concentrations of Cys. (B) Proteomic assay of the relative level of proteins associated with cysteine metabolism in *P. stutzeri* 273 under LB alone and LB with 8 mmol/L L-cysteine. (C) The in-gel activity assay with bismuth staining applied for detecting the proteins that produced H₂S from L-cysteine in *P. stutzeri* 273 wild type (PS273), mutant strains ($\Delta CSE1$, $\Delta CSE2$, $\Delta CSE1\Delta CSE2$, ΔTD), and the psTD encoding gene complementary strain $\Delta TD/cTD$. Lane 1: PS273, Lane 2: $\Delta CSE1$, Lane 3: $\Delta CSE2$, Lane 4: $\Delta CSE1\Delta CSE2$, Lane 5: ΔTD , Lane 6: $\Delta TD/cTD$. (D) H₂S production of PS273 and its mutant strains incubated in LB supplemented with different concentrations of Cys for 12 h.

Fig. 2 Products determination of L-cysteine catalyzed by psTD. (A) Absorbance spectrum of purified psTD in Tris buffer (50 mmol/L Tris, 150 mmol/L NaCl, 250 mmol/L imidazole, pH 8.0) (black line) and unbound pyridoxal 5'-phosphate (PLP) (0.02 mmol/L in Tris buffer, red line). Insert in panel A: SDS-PAGE gel (left) of purified psTD (20 µg) showing a size around 40 kDa and Native-PAGE gel (right) with bismuth staining for 30 min showing psTD (0.4 µg) with the ability of catalyzing H₂S generation from L-cysteine. (B) LC-MS analysis of the reaction products catalyzed by psTD. (C) Determination of pyruvate concentration in the enzymatic mixture catalyzed by psTD. (D) The proposed reaction of L-cysteine catalyzed by psTD.

Fig. 3 Structure comparison of different psTD crystal structures. (A) PLP bound to K51 with possible covalent bond and covalent bond in chain A and chain B of structure 7DAP (carbon in purple for key residues), respectively. (B) An amino acid (e.g. cysteine) bound to K51 in chain A of structure 7DAQ (two NH_4^+ , carbon in cyan for key residues); PLP bound to K51 with non-covalent bond in chain B of 7DAQ (one NH_4^+ , R77 extending orientating outside for both chain A and B). (C) Structure comparison of 7DAQ and 7DAP for chain A (one NH_4^+ overlapping with PO_4^- of the virtually corresponding PLP) and for chain B. (D) SDS-PAGE gel and Native-PAGE gel of purified psTD wild type (R) and variants R77A (A), R77E (E), and R77K (K) with bismuth staining (without or with 2 mol/L NH_4^+) for 5 min. The loading amount of each lane is 9 μg . (E) Dual conformation of E77 in chain A (7DAR, carbon in cyan for key residues, carbon in orange for alternative E77); Dual conformation G78 in chain B (7DAR, with the virtually corresponding PLP, carbon in orange for alternative G78).

Fig. 4 The map of electrostatic potential (MEP) comparison of the inside active sites from different psTD structures. (A) MEP inside of structure 7DAP (chain A and B, carbon in cyan). (B) MEP of 7DAQ and key residues comparison for 7DAQ (carbon in orange) and 7DAP (chain A and B). (C) MEP of 7DAR and key residues comparison for 7DAR (carbon in green), 7DAP and 7DAQ (chain A and B).

Fig. 5 The molecular interactions between PLP and psTD near the active site. (A) The polar interactions between PLP and psTD structure (chain A and B of 7D8Y, the same as below, dashed line indicates the possible hydrogen bonds). **(B)** The PLP contacting residues of psTD (chain A). **(C, D)** 2D plot of the interactions between PLP and psTD (chain A and B). **(E)** Weblogo for the amino acids around K51, R77 and G177-G181. The size of a single letter amino acid code in the sequence logo represents the occurrence of a particular amino acid at a particular position. The numbers are the locations of amino acids in psTD. psTD sequence is underneath of the horizontal axis.

Fig. 6 The detailed comparison of active sites from psTD and 1TDJ. (A) Key residues comparison around PLP between psTD (chain B of 7D8Y, green carbon) and 1TDJ (purple carbon). (B) Pockets of active site from psTD with surrounding residues interacting PLP. (C) MEP of psTD (surface) and 1TDJ (mesh). (D), (E), (F) MEP of psTD (mesh) and 1TDJ (surface) from three different views. The access channel (or gate) for active site of 1TDJ is circled with red dashline in (E).

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585 **Fig. 7 Three pockets of active site of psTD structure.** Pocket I (occupied by PLP),
 586 Pocket II (for reaction products/small molecules), and Pocket III (access channel) of
 587 active site from psTD (chain B of 7D8Y). (A) Key residues interacting with PLP at
 588 Pocket I (green circle, residues in green carbon). (B) Key residues of Pocket I and II
 589 (deep purple circle, residues in deep purple carbon). Key residues of Pocket I and II
 590 (R77 inward orientation) (C), and together with Pocket III (brown circle, residues in
 591 brown carbon) (R77 outward orientation), comparing with Pockets I and II MEP in

592 mesh (**D**). Notes: Pocket I and II with R77 inward orientation shown as MEP surface
 593 in A, B and C; R77 outward orientation shown in D; both R77 inward and outward
 594 conformations compared and shown rotation angle in C. (**E**) Proposed mechanisms of
 595 structure dynamics and specificity for psTD enzyme activities: R77 switch states
 596 between inward and outward orientation, which results in PIII open or close for small
 597 molecule exchange and corresponding reactions.

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