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Characterization of a novel moderate-substrate specificity amino acid racemase from the hyperthermophilic archaeon *Thermococcus litoralis*

ARCHAEAL AMINO ACID RACEMASE

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Abstract

The amino acid sequence of the OCC_10945 gene product from the hyperthermophilic archaeon *Thermococcus litoralis* DSM5473, originally annotated as γ-aminobutyrate aminotransferase, is highly similar to that of the uncharacterized pyridoxal 5′-phosphate (PLP)-dependent amino acid racemase from *Pyrococcus horikoshii*. The OCC_10945 enzyme was successfully overexpressed in *Escherichia coli* by co-expression with a chaperone protein. The purified enzyme demonstrated PLP-dependent amino acid racemase activity primarily toward Met and Leu. Although PLP contributed to enzyme stability, it only loosely bound to this enzyme. Enzyme activity was strongly inhibited by several metal ions, including Co^{2+} and Zn^{2+}, and non-substrate amino acids such as L-Arg and L-Lys. These results suggest that the underlying PLP-binding and substrate recognition mechanisms in this enzyme are significantly different from those of the other archaeal and bacterial amino acid racemases. This is the first description of a novel PLP-dependent amino acid racemase with moderate substrate specificity in hyperthermophilic archaea.

Keywords: pyridoxal 5′-phosphate, amino acid racemase, substrate specificity, *Thermococcus litoralis*

Introduction

In recent years, the presence of various D-amino acids has been reported in many microorganisms, animals, and plants (Kobayashi 2020). Amino acid racemases, which catalyze the reversible conversion of L-amino acids to D-amino acids, are thought to be the major force behind the production of D-amino acids. Amino acid racemases are divided into two groups: pyridoxal 5′-phosphate (PLP)-dependent and PLP-independent enzymes. Alanine racemase (AlaR), methionine racemase, and serine racemase (SerR) are all members of the former group, whereas aspartate racemase (AspR), glutamate racemase (GluR), and proline racemase (ProR) are all members of the latter group (Yoshimura *et al.* 2003; Radkov *et al.* 2014). In bacteria, AlaR and GluR, which exhibit high substrate specificity, are known to function in the synthesis of D-Ala and D-Glu, respectively, both of which form part of the peptidoglycan layer in the cell wall (Hernández *et al.* 2016). Several reports have described the presence of PLP-dependent amino acid racemases with broad substrate specificity (BAR) in several bacterial species, including *Pseudomonas putida* and *Lactobacillus buchneri* (Lim *et al.* 1993; Mutaguchi *et al.* 2013). Several free D-amino acids have also been reported in archaeal species, and PLP-independent AspR and ProR, and PLP-dependent AspR and SerR have been characterized as archaeal racemases (Matsumoto *et al.* 1999; Nagata *et al.* 1999; Long *et al.* 2001; Liu *et al.* 2002; Ohnishi *et al.* 2008; Watanabe *et al.* 2015; Washio *et al.* 2016).

When we investigated the relationship between D-amino acid utilization in a hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3 and its cell growth, we found

that *P. horikoshii* exhibited slow or no growth in the absence of the L-forms of Met, Leu, Val, Ile, Phe, Tyr, Trp, or Arg; however, the cell growth was recovered by the addition of the D-forms of these amino acids (Kawakami *et al.* 2015). During this analysis, we detected amino acid racemase activity in the crude extracts of *P. horikoshii*, partially purified the enzyme, and identified the coding gene (PH0138) (Kawakami *et al.* 2015). The recombinant enzyme had a dimer structure with a subunit molecular mass of 52.1 kDa and racemase activity for Phe, Leu, Met, Tyr, Ile, Thr, Trp, Val, Ser, and Ala. This indicates that this enzyme is a typical BAR and is likely responsible for the utilization of D-amino acids during *P. horikoshii* growth (Kawakami *et al.* 2015).

The PH0138 gene was originally annotated as γ-aminobutyrate aminotransferase (GABA-AT). The genome database includes three other GABA-AT genes, PH0782, PH1423, and PH1501, which share more than 45% sequence identity with each other. We have recently revealed that the hexamer of the PH0782 gene product (50.5 kDa) exhibits Ala- and Ser-specific racemase (ASR) activity (Kawakami *et al.* 2018). We also detected ornithine aminotransferase (OrnAT) activity, but no amino acid racemase activity, in the tetramer structure of the PH1423 gene product (50.6 kDa) (unpublished results). It has been reported that the TK2101 gene product from *Thermococcus kodakarensis*, which shares nearly 60% of its sequence identity with PH1423, also exhibits OrnAT activity (Zheng *et al.* 2018).

We had previously reported that the PH1501 gene product (48.0 kDa) displays low racemase activity toward Met, Phe, and Leu, suggesting that the substrate specificity of this enzyme differs from those of both BAR and ASR (Kawakami *et al.* 2018).

However, it did not show up as a sharp band on native-polyacrylamide gel electrophoresis (PAGE), unlike other GABA-AT proteins (Fig. 1A), suggesting a lack of uniformity in this protein molecule. In addition, this enzyme was easily inactivated during dialysis, indicating that the protein is not suitable for purification and characterization. To demonstrate the presence of novel archaeal amino acid racemases, we constructed an expression plasmid for the OCC_10945 gene from *T. litoralis* DSM5473, whose product shares 75.5% sequence identity with the PH1501 gene product, overexpressed this enzyme using a chaperone-dependent co-expression system, and determined its enzymatic properties.

Materials and methods

Materials

Escherichia coli BL21(DE3) and BL21(DE3) CodonPlusRIPL competent cells were purchased from Agilent Technologies Japan (Tokyo, Japan). PrimeSTAR Max DNA polymerase and the chaperone plasmids were purchased from Takara Bio (Shiga, Japan). *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-m-toluidine (EHSPT), 4-aminoantipyrin (4-AA), *o*-phthalaldehyde (OPA), *N*-*tert*-butyloxycarbonyl-L-cysteine (NBC), and horseradish peroxidase (HRP) were purchased from Wako Pure Chemicals (Tokyo). D-Amino acid oxidase (DAO) from porcine kidney was obtained from Sigma-Aldrich Japan (Tokyo). All other reagents were of analytical grade and purchased from commercial sources.

106 ***Expression plasmid construction***

107 The OCC_10945 expression plasmid was constructed using the same construction
108 scheme used to produce the PH1501 expression plasmid (Kawakami *et al.* 2018).
109 Polymerase chain reaction (PCR) using the oligonucleotides (OCC_10945-NdeI:
110 CCATATGAGAAAGGAGGAAATTATAGAGGG and OCC_10945-BamHI:
111 TGGATCCCTACCAGCCGTGAACAAAC) and *T. litoralis* genomic DNA was used to
112 produce an insert for the OCC_10945 plasmid. A silent mutation in the NdeI recognition
113 sequence within the gene was introduced using oligonucleotides (OCC_10945-MutFw:
114 AGAGGGCTTATGATGATGAAGCCAAGAAG and OCC_10945-MutRv:
115 CATCATAAGCCCTCTCTTTAGTCTCC). PrimeSTAR Max DNA polymerase was
116 used for both the PCR amplification and silent mutation of the OCC_10945 gene
117 according to the manufacturer's instructions. The gene sequence of the resultant
118 expression plasmid, pET15b/OCC_10945, was confirmed using a Model 3130 Genetic
119 Analyzer (Applied Biosystems, Tokyo, Japan).
120
121 ***Co-expression with chaperone plasmids and purification of the recombinant enzyme***

122 *Escherichia coli* BL21 (DE3) competent cells harboring chaperone plasmids (pG-
123 KJE8, pGro7, pKJE7, pG-Tf2, and pTf16) were produced according to the
124 manufacturer's instructions, and the resultant competent cells were further transformed
125 with pET15b/OCC_10945. Transformants harboring each chaperone plasmid were
126 separately cultivated in Luria-Bertani medium containing 100 μg mL^{-1} ampicillin, 20 μg
127 mL^{-1} chloramphenicol, and appropriate inducers (0.5 mg mL^{-1} L-arabinose for *ara*B

and/or 5 ng mL^{-1} tetracycline for *Pzt*-1) for 9 h at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the medium to a final concentration of 0.5 mM, and the cells were left to grow for another 3 h before they were harvested by centrifugation and stored at -20 °C until use.

To purify the recombinant enzyme, the cells were suspended in 10 mM Tris/HCl (pH 8.0), treated with lysozyme, disrupted by sonication, and centrifuged. The resultant crude extract was then mixed with 0.1 mM PLP and heat-treated at 90 °C for 30 min, cooled on ice, and centrifuged to remove precipitates. Imidazole and NaCl were added to the resultant enzyme solution at concentrations of 0.01 and 0.5 M, respectively, and this solution was applied on a Ni-Chelating Sepharose (Cytiva, Tokyo) column previously equilibrated with 10 mM Tris/HCl (pH 8.0) supplemented with 0.01 mM imidazole and 0.5 M NaCl. The column was then washed with the same buffer, and the enzyme was eluted using a 0.01-0.5 M imidazole gradient. Active fractions were pooled and dialyzed with 10 mM Tris/HCl (pH 8.0) and used for characterization.

Determination of enzyme activity, enzymatic properties, and protein concentration

The racemase activities for Met, Leu, Phe, and Ala were assayed using a spectrophotometric method, as previously described (Kawakami *et al.* 2015). The first reaction mixture (0.1 mL) was composed of 100 mM Tris/HCl (pH 8.0), 10 mM L-amino acid, and 0.2 mM PLP. The enzyme was incubated in this solution at 80 °C for an appropriate length of time. After cooling on ice, the second reaction mixture (0.1 mL) containing 100 mM Tris/HCl (pH 8.0), 0.4 mM 4-AA, 0.6 mM EHSPT, 0.2 U mL^{-1}

150 DAO, and 4 U mL^{-1} HRP was added to the first reaction mixture, and then incubated at
151 37 °C for 30 min. The increase in absorbance at 555 nm, following the production of
152 quinonimine dye, was then measured using a U-1900 spectrophotometer (Hitachi High-
153 Tech Science, Tokyo). The amount of D-amino acid produced by each racemase reaction
154 was calculated using the calibration curve for each D-amino acid. The enzyme activity
155 was represented as the amount of D-amino acid (μmol) produced in 1 min at 80 °C.
156 To investigate the effect of PLP on enzyme stability, the enzyme was incubated at
157 40–100 °C for 20 min or at 80 °C for 0–7 h in the absence and presence of 0.2 mM PLP,
158 and the residual activity was then determined under standard assay conditions. To
159 determine the pH dependency of the racemase reactions, each reaction was performed at
160 80 °C for 20 min using different buffers (50 mM). After cooling, the mixtures were
161 diluted twice with 50 mM Tris/HCl (pH 8.0) for neutralization and then subjected to a
162 second reaction. To determine pH stability, the residual activity was determined under
163 standard assay conditions after incubation of the enzyme in 100 mM buffers at 60 °C for
164 20 min in the presence of 0.2 mM PLP. The buffers used in the pH dependency and
165 stability analysis can be described as follows: acetate/sodium acetate (pH 3.5 to 6.5),
166 citrate/sodium citrate (pH 3.5 to 6.0), MES/NaOH (pH 5.0 to 7.0), Bis-Tris/HCl (pH 5.5
167 to 7.5), KH_2PO_4/K_2HPO_4 (pH 6.0 to 8.0), HEPES/NaOH (pH 6.5 to 8.5), Tris/HCl (pH
168 7.0 to 8.5), glycine/NaOH (pH 8.5 to 10.5), and K_2HPO_4/NaOH (pH 11.0 to 12.5).
169 Enzyme assays in the presence of 1 mM hydroxylamine HCl, 1 mM EDTA, 1 mM
170 metal ions ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnSO_4$), and non-
171 substrate amino acids (10 mM L-Asp, L-Glu, Gly, L-His, L-Ile, L-Lys, L-Asn, L-Pro, L-

172 Gln, L-Arg, L-Thr, and L-Trp, and 2 mM L-Tyr) were designed to determine the effects
173 of various inhibitors on the activity of OCC_10945. In these assays, L-Met was used as
174 the substrate.
175 Ultra-performance liquid chromatography (UPLC) was used to determine the
176 substrate specificity of this enzyme. Thirteen D-amino acids produced from L-amino
177 acids by the enzyme reaction were derivatized using OPA and NBC and analyzed by
178 reverse phase chromatography using an X-pressPak V-C18 column (Kawakami *et al.*
179 2017). Kinetic analysis was also performed using this system. HEPES/NaOH (pH 7.0)
180 was used as the reaction buffer, and the reaction was performed at several
181 concentrations of L- and D-Met (1–40 mM) and L- and D-Leu (1–20 mM). These data
182 were analyzed using a non-linear regression model in Prism 5.0 (GraphPad Software).
183 Protein concentrations were determined using the Bradford method (1976), with
184 bovine serum albumin serving as the standard.
185
186 ***Polyacrylamide gel electrophoresis***
187 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was
188 performed using the Laemmli method (1970). Precision plus protein standards were
189 used as molecular mass standards (Bio-Rad, Tokyo). Native-PAGE was performed using
190 the method described by Davis (1964).
191
192 ***Phylogenetic analysis***

193 The amino acid sequences of the Thermococcales GABA-ATs were collected from
194 the KEGG database (https://www.genome.jp/kegg/) and aligned using ClustalW
195 multiple sequence alignment software (ver. 2.1) via the DNA Data Bank of Japan
196 (DDBJ) website (https://www.ddbj.nig.ac.jp/index.html). A phylogenetic tree was
197 constructed using the neighbor-joining (NJ) method and visualized using Molecular
198 Evolution Genetic Analysis (MEGA) software package.
199
200 **Results and discussion**
201 ***Phylogenetic analysis of the Thermococcales GABA-AT genes***
202 We showed that there are four putative GABA-AT genes within the *P. horikoshii*
203 genome (Kawakami *et al.* 2015). These gene products are classified as aminotransferase
204 class III within the fold-type I PLP-enzyme. We observed that similar GABA-AT type
205 genes were conserved across all of the Thermococcales order. We collected the primary
206 sequences of GABA-ATs (91 sequences in 35 strains) from the KEGG database,
207 analyzed them using ClustalW, and constructed a phylogenetic tree using MEGA (Fig.
208 2). Interestingly, the BAR enzyme encoded by the PH0138 gene grouped with only two
209 other enzymes (encoded by PAB0086 from *P. abyssi* and PNA2_0667 from *Pyrococcus*
210 sp. NA2), while the Orn-AT enzymes were present in all of the characterized strains
211 (Supplementary Table S1). The PH1501 gene formed another cluster along with the
212 other 31 homologous genes evaluated in this study.
213 Three genes annotated as GABA-AT (OCC_00582, OCC_08410, and OCC_10945)
214 were also found to be conserved within the genome of *T. litoralis*; the first two genes

were classified as OrnAT and ASR, respectively. These recombinant enzymes produced by *E. coli* exhibited appropriate OrnAT and ASR activities (data not shown). This indicates a strong correlation between the phylogenetic position and the enzyme activity of these proteins, suggesting that GABA-AT genes could be classified based on their phylogenetic analysis alone. Taken together, these data suggest functional similarities between OCC_10945 and PH1501 enzymes.

Co-expression of OCC_10945 gene with chaperones

We first used *E. coli* BL21(DE3) CodonPlusRIPL cells to overexpress the OCC_10945 gene, similar to our approach for other GABA-AT genes (Kawakami *et al.* 2017 and 2018). However, almost all of the recombinant protein was produced as inclusion bodies and no amino acid racemase activity was detected in the soluble fraction following heat treatment (80 °C for 20 min) of the crude extract. No improvement was achieved by induction at lower temperatures or changes in the host cells (data not shown).

Next, we overexpressed this gene in a co-expression system with a chaperone. Fig. 1B shows the SDS-PAGE analysis of the soluble fractions after heat treatment of the crude extracts and inclusion bodies from the systems. When the OCC_10945 gene was expressed in conjunction with the pGro7 chaperone plasmid, the recombinant protein could be produced in the soluble fraction, allowing for the evaluation of its activity. A high degree of amino acid racemase activity for L-Met was also detected in the soluble fraction following heat treatment (Fig. 1C). This indicates that the GroES-GroEL

237 chaperone assists in the proper folding of the OCC_10945 gene product. Thus, we
238 selected this co-expression system for large-scale preparation of this recombinant
239 enzyme.
240 The recombinant enzyme was successfully purified using heat treatment and Ni-
241 Chelating Sepharose chromatography, with no precipitation during the dialysis
242 procedure (Fig. 1D, right). Unlike PH1501, the OCC_10945 enzyme was successfully
243 detected as a band with a mobility similar to that of PH0138 on native-PAGE (Fig. 1A
244 and D). However, we could not determine the molecular mass of this enzyme because it
245 did not produce a clear peak during gel filtration chromatography through a UP
246 SW3000 column (Tosoh, Tokyo) (data not shown).
247
248 ***Characterization of OCC_10945 enzyme***
249 Similar to PH1501, the OCC_10945 enzyme exhibited racemase activity for Met,
250 Leu, and Phe. The specific activities of this enzyme for these substrates at 10 mM were
251 determined to be approximately 1.38, 1.08, and 0.34 μmol min^{-1} mg^{-1}, respectively.
252 These specific activities were similar to those of the PH1501 enzyme and were more
253 than 10 times lower than those of PH0138 (Kawakami *et al.* 2017).
254 We also demonstrated that the OCC_10945 enzyme had no detectable activity in the
255 absence of PLP. The activity was shown to increase with increasing PLP concentrations,
256 and the K_m value for PLP was determined to be approximately 28 μM (Fig. 3A). The
257 activity was also blocked by the addition of 1 mM hydroxylamine, which is known to
258 inhibit the reaction of PLP-enzymes (Table 1). This indicates that PLP acts as a

coenzyme for this enzyme but does not tightly bind to the OCC_10945 enzyme. Both BAR and ASR were shown to be active even in the absence of PLP in the reaction mixture, and PLP was not released from either enzyme during purification or dialysis (Kawakami *et al.* 2017 and 2018). This suggests that the underlying PLP-binding mechanism of the OCC_10945 enzyme is totally unique from those of both BAR and ASR. We also observed that the addition of PLP increased the thermostability of the enzyme (Fig. 3B and C). The OCC_10945 enzyme retained 80% of its activity after incubation at 80 °C for 7 h in the presence of PLP, indicating that the thermostability of this enzyme is comparable to those of BAR and ASR, which are stable at 80 °C for several hours (Kawakami *et al.* 2017 and 2018). On the other hand, this enzyme was unstable at pH values below 7.0 and had its highest activity at temperatures between 70 and 80 °C or acetate buffer (pH 4.5) (Fig. 3D, E, and F). These results demonstrate that there are distinct differences in the stabilities and dependencies of the temperature and pH of this enzyme versus those of the BAR and ASR enzymes. For example, BAR and ASR prefer neutral pH values (approximately 6.5–7.5) for their reactions (Kawakami *et al.* 2017 and 2018). This could be due to the differences in the catalytic sites of these enzymes.

We evaluated substrate specificity using 13 L-amino acids. UPLC analysis revealed that the OCC_10945 enzyme could catalyze the racemase reaction of Met, Leu, Phe, Ala, and Ser. However, Val, Ile, Asn, Thr, Gln, Arg, Tyr, and Trp were inert as substrates. In addition, the spectrophotometric assay revealed that this enzyme could

also catalyze the racemase reaction of nor-Leu, nor-Val, and 2-aminobutyrate, comparable to Met and Leu (data not shown).

Next, we determined the kinetic parameters for the L- and D-forms of Met and Leu using non-linear regressions (Table 2). The k_{cat} values for both D- and L-Met were higher than those for D- and L-Leu, respectively, while the K_m values for D- and L-Met were more than three times higher than those for D- and L-Leu. Thus, the resulting catalytic efficiencies (k_{cat}/K_m) indicate that Leu is the preferred substrate for this enzyme. Taken together, these results indicate that the OCC_10945 enzyme is a novel archaeal amino acid racemase with a unique substrate specificity differentiating it from both BAR (PH0138) and ASR (PH0782).

We then determined the effect of potential inhibitors, such as metal ions and non-substrate amino acids, on enzyme activity. It has already been reported that Ile 2-epimerase (ILEP) from *L. buchneri*, which shares 43% sequence identity with the OCC_10945 enzyme, shows no inhibition by metal ions at a concentration of 10 mM (Mutaguchi *et al.* 2013). In contrast, Co^{2+}, Cu^{2+}, Fe^{3+}, Ni^{2+}, and Zn^{2+} ions at a concentration of 1 mM strongly inhibited OCC_10945 enzyme activity (Table 1). In addition, the activity was also inhibited by non-substrate amino acids, Arg, Lys, His, Ile, and Trp at 10 mM, especially Arg and Lys. The amine group side chains of these amino acids may be responsible for this inhibition. In general, Ala, Val, Ile, and Leu are classified as hydrophobic amino acids with side chains of different lengths. It has been reported that BAR and Ile 2-epimerase can exhibit activity for these hydrophobic amino acids depending on the length of the side chain (Mutaguchi *et al.* 2013; Kawakami *et al.*

2017). The OCC_10945 enzyme exhibited no activity for Ile and Val regardless that Leu and Ala were applicable as substrates. Moreover, L-Ile inhibited the activity (Table 1). Further evaluations of the relationships between function and structure will be necessary to understand the detailed inhibition mechanism of these non-substrate amino acids.

In summary, GABA-AT genes from Thermococcales archaea can be classified into four functional groups, with three of these groups exhibiting amino acid racemase activity. The first group is BAR, which exhibits the activities toward a wide variety of substrates, including hydrophobic and aromatic amino acids. The second group includes the ASR, which exhibits activities toward Ala and Ser. Here, we characterized the OCC_10945 enzyme and identified this enzyme as a novel type of amino acid racemase. This enzyme exhibited neither specific nor broad substrate preference. Thus, we refer to this type of racemase as a moderate substrate specificity amino acid racemase, MAR. We are in the process of preparing and comparing the crystal structures of all three types of amino acid racemases and hope that this may shed light on the differences in their activity and specificity. These studies will provide useful insights into the subunit structure, PLP binding, and metal and amino acid inhibition.

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Author contribution

R.K. designed the study. C.K., T.K., and M.S. performed the experiments, including gene cloning, protein expression and purification, and enzyme characterization. R.K. wrote the original draft of the manuscript, and J.H., H.S., and T.O. reviewed and edited the original draft.

Disclosure statement

The authors have no potential conflict of interest to declare.

Statement of ethics

This research did not require ethical approval.

Data availability

All data are incorporated into the article and its online supplementary material.

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414
415

Figure Legends

Fig. 1 Expression and purification of the OCC_10945 gene product

A) Native-PAGEs of *P. horikoshii* GABA-AT homologs using disc gel electrophoresis. These recombinant enzymes were partially purified using heat treatment and a Ni chelating column. B) SDS-PAGE analysis of the OCC_10945 production when using each co-expression system. The supernatants after heat treatment and the inclusion bodies were loaded on the left and right sides of the gel, respectively. C) Specific activities of the heat-treated enzymes. L-Met was used as the substrate in the assay (n = 3). D) Analyses of the purified OCC_10945 using native (left) and SDS-PAGEs (right).

Fig. 2 Phylogenetic tree of GABA-AT sequences from various Thermococcales strains

The GABA-ATs from *T. litoralis* and *P. horikoshii* are marked by stars and diamonds, respectively. Abbreviations are as follows: pfu, *P. furiosus*; pho, *P. horikoshii*; pab, *P. abyssi*; pyn, *Pyrococcus* sp. NA2; pya, *P. yayanosii*; pys, *Pyrococcus* sp. ST04; pyc, *P. kukulkanii*; tko, *T. kodakarensis*; ton, *T. onnurineus*; tga, *T. gammatolerans*; tsi, *T. sibiricus*; tba, *T. barophilus*; the, *Thermococcus* sp. 4557; tha, *Thermococcus* sp. AM4; thm, *T. cleftensis*; tlt, *T. litoralis*; ths, *T. paralvinellae*; tnu, *T. nautili*; teu, *T. eurythermalis*; tgy, *T. guaymasensis*; thv, *Thermococcus* sp. 2319×1; tch, *T. chitonophagus*; tpep, *T. peptonophilus*; tpie, *T. piezophilus*; tgg, *T. gorgonarius*; tce, *T. celer*; tbs, *T. barossii*; thh, *Thermococcus* sp. 5-4; tsl, *T. siculi*; ttd, *T. thioreducens*; tprf, *T. profundus*; trl, *T. radiotolerans*; tpaf, *T. pacificus*; thy, *Thermococcus* sp. P6; and

ppac, *Palaeococcus pacificus.* The GABA-ATs were classified into four groups (Groups 1 to 4) based on the recommendations of Kawakami *et al.* (2015).

Fig. 3 Characterization of the OCC_10945 enzyme

A) Effect of PLP concentration on enzyme activity. B) Enzyme stability at various temperatures (20 min incubation) in the absence (open circles) and presence (closed circles) of 0.2 mM PLP. C) Stability of enzyme at 80 °C for various incubation times in the absence (open circles) and presence (closed circles) of 0.2 mM PLP. D) Enzyme stability after incubation at various pH values at 60 °C for 20 min in the presence of 0.2 mM PLP. In B, C, and D, the residual activity (100 %: activity of enzyme without incubation) was determined under the standard assay conditions. E) Effect of temperature on enzyme activity. The activity at 80 °C was defined as 100 %. F) Effect of pH on enzyme activity. The activity using Tris/HCl (pH 8.0) was defined as 100 %. The buffers used in D and F are as follows: closed circle: acetate/sodium acetate (pH 3.5 to 6.5); open circle: citrate/sodium citrate (pH 3.5 to 6.0); open triangle: MES/NaOH (pH 5.0 to 7.0); closed diamond: Bis-Tris/HCl (pH 5.5 to 7.5); closed square: KH_2PO_4/K_2HPO_4 (pH 6.0 to 8.0); open square: HEPES/NaOH (pH 6.5 to 8.5); open diamond: Tris/HCl (pH 7.0 to 8.5); closed triangle: glycine/NaOH (pH 8.5 to 10.5); and open triangle: K_2HPO_4/NaOH (pH 11.0 to 12.5).

460 **Graphical abstract caption**

461

462 The GABA-ATs classified into group 4 in Thermococcales order of archaea were

463 identified as PLP-dependent moderate-substrate specificity amino acid racemase.

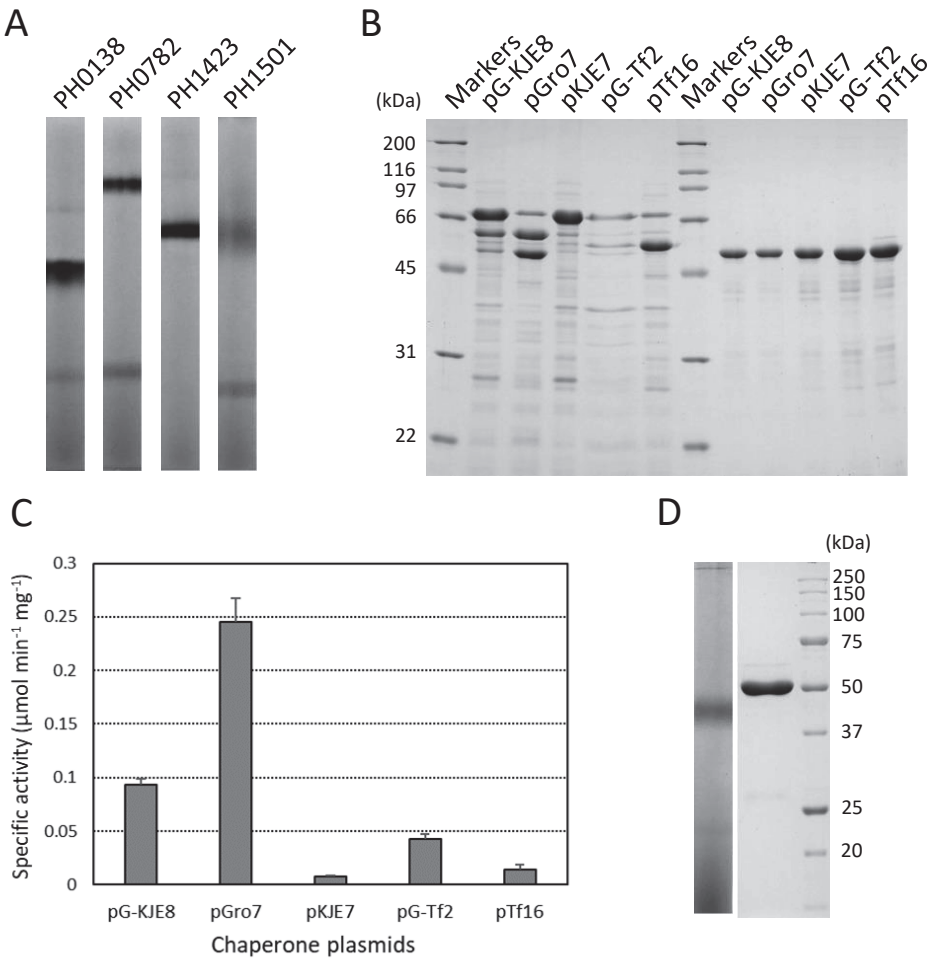

Fig 1 (Kawakami et al)

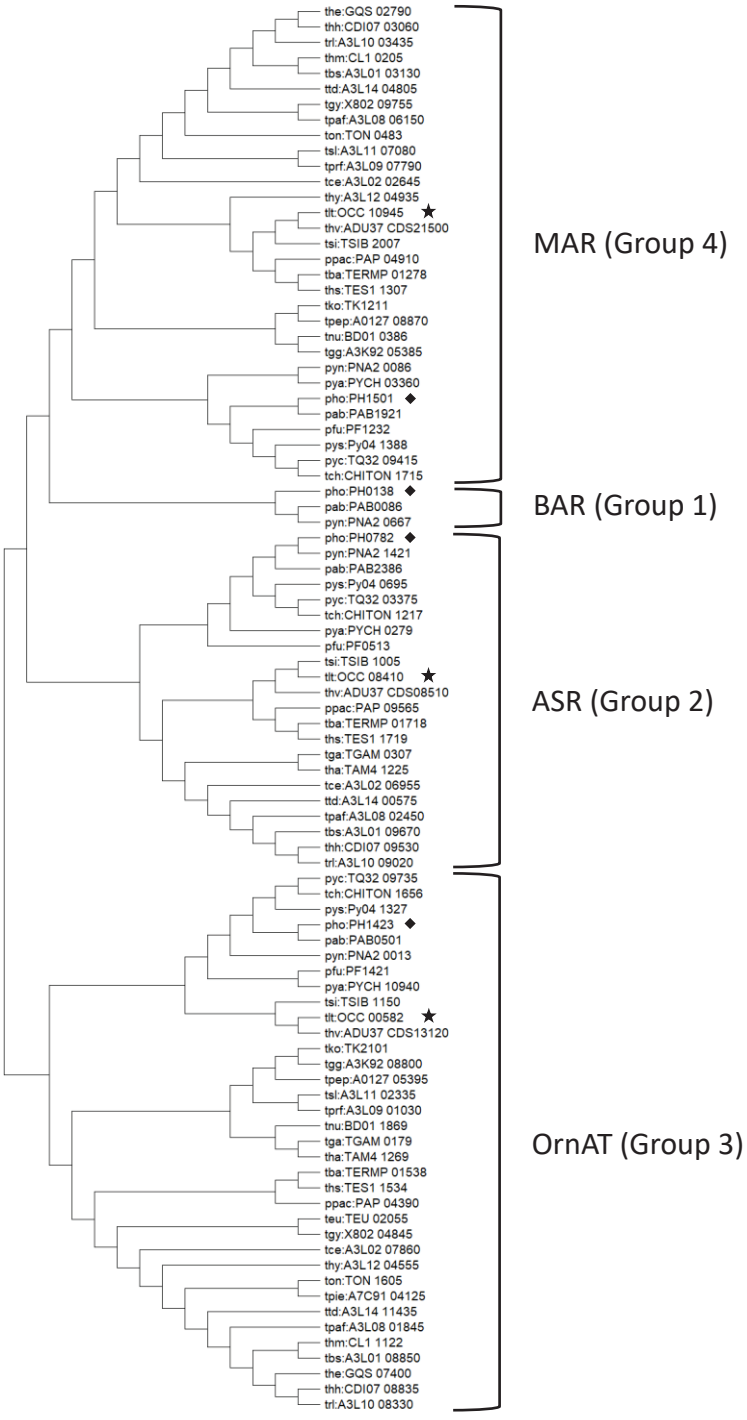

Fig 2 (Kawakami et al)

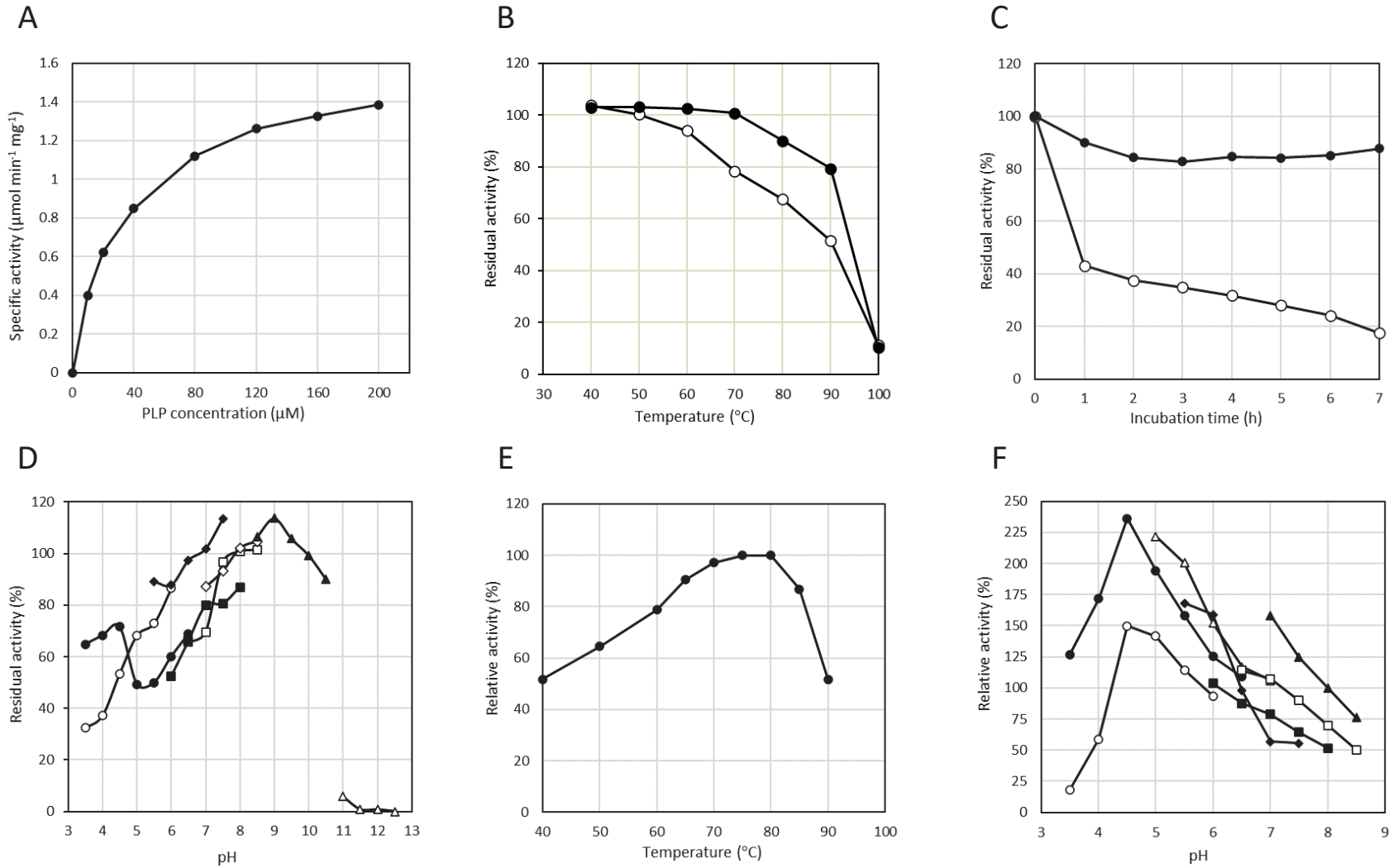

Fig 3 (Kawakami et al)

Table 1 Effects of chemicals on the OCC_10945 enzyme activity

Chemicals [a]	Residual activity (%) [b]	Chemicals [a]	Residual activity (%) [b]
Hydroxylamine	7	Gly	97
EDTA	109	L-His	55
$CaCl_2$	97	L-Ile	55
$CoCl_2$	6	L-Lys	26
$CuCl_2$	20	L-Asn	83
$FeCl_3$	35	L-Pro	94
$NiCl_2$	16	L-Gln	103
$MgCl_2$	86	L-Arg	4
$MnCl_2$	60	L-Thr	98
$ZnSO_4$	7	L-Val	97
L-Asp	98	L-Trp	50
L-Glu	100	L-Tyr	95

[a] Concentrations of each chemical used in the assay are shown in the Materials and Methods.

[b] Enzyme activity was determined in the presence and absence of each chemical (n = 3), and the residual activities were calculated.

Table 2 Kinetic parameters of the OCC_10945 enzyme

	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($sec^{-1}mM^{-1}$)
L-Met	1.1 ± 0.026	6.7 ± 0.50	0.16
D-Met	1.8 ± 0.071	12 ± 1.1	0.14
L-Leu	0.62 ± 0.013	2.3 ± 0.15	0.28
D-Leu	0.79 ± 0.015	1.8 ± 0.11	0.44

Enzyme activities were determined at various substrate concentrations, as described in the Materials and Methods (n = 3).