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ORIGINAL RESEARCH PAPER

A novel SGNH family hydrolase Ali5 with thioesterase activity and a GNSL motif but without a classic GDSL motif from *Altererythrobacter ishigakiensis*

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Abstract
Objective We aimed to characterize a novel SGNH (Ser-Gly-Asn-His) family hydrolase from the annotated genome of marine bacteria with new features.
Results A novel esterase Ali5 from *Altererythrobacter ishigakiensis* has been identified and classified into SGNH family. Ali5 presented a novel GNSL (Gly-Asn-Ser-Leu(X)) motif that differs from the classic GDSL (Gly-Asp-Ser-Leu(X)) motif of SGNH family. The enzyme has esterase and thioesterase activity and exhibited apparent temperature and pH optima of 40 °C and pH 7.5 (in phosphate buffer), respectively. Ali5 was found to be halotolerant and thermostable, and exhibited strong resistance to several organic solvents and metal ions. The residue Tyr^{196} has a great influence on the catalytic activity, which was proved by site-directed mutagenesis and subsequent kinetic characterization.
Conclusion The esterase Ali5 with esterase and thioesterase activities, salt and metal ions resistance and unique structural features was identified, which holds promise for research on the SGNH family of hydrolases.

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Keywords *Altererythrobacter ishigakiensis* NBRC 107699^T · GNSL motif · SGNH family esterase · Substrate binding cavity · Thioesterase

Introduction

Bacterial lipolytic enzymes have been studied extensively due to their excellent catalytic properties and broad applications in biotechnology. These lipolytic enzymes have been classified into eighteen families (Samoylova et al. 2018). The SGNH hydrolase superfamily has attracted much attention due to the unique structural features and catalytic mechanisms of its members. In contrast to other bacterial lipolytic enzyme families, the SGNH hydrolase superfamily contains five conserved blocks (I–V). A conserved Gly-Asp-Ser-Leu(X) motif in block I contains the active site serine residue near the N-terminus. Moreover, four invariant residues (Ser, Gly, Asn, His) are distributed in blocks I, II, III and V, respectively (Akoh et al. 2004). Normally, the Gly (block II) and Asp (block IV) residues constitute an oxyanion hole, and residues Ser (block I), His and Asp/Glu (block IV) constitute the catalytic triad, but some of SGNH hydrolases lack the Asp/Glu and therefore form a catalytic dyad (Lescic Asler et al. 2017).

Based on these unique structural features, SGNH family hydrolases possess broad substrate specificity and multi-functionality (Lescic Asler et al. 2010). Moreover, the hydrolases of the SGNH family have also been reported excellent salt tolerance, organic solvents tolerance, cold adaptability, and thermostability. Their ability to perform catalytic functions under harsh conditions makes them highly attractive biocatalysts for the food processing, flavor and fragrances, cosmetic, medical, and laundry detergent industries (Akoh et al. 2004; Farn et al. 2001; Fucinos et al. 2012).

The ocean possesses very complex and varying environmental conditions of temperature, pH, pressure, metal ions, and salinity (Kennedy et al. 2008). Therefore, enzymes from marine microorganisms distributed in hydrothermal vents, metallic sulfide areas and seafloor sediments are expected to possess specific catalytic properties and structural features (Rong et al. 2018; Wu et al. 2015).

In this study, Ali5, a new member of the SGNH hydrolase family, was cloned from the annotated genome of the *A. ishigakiensis* NBRC 107699^T which was isolated from a marine sediment, and then was expressed, and characterized in detail. Moreover, the influence of residue Asp^{55} and Tyr^{196} to catalytic activity was investigated.

Materials and methods

Strains, vector, media, and culture conditions

Altererythrobacter ishigakiensis NBRC 107699^T was purchased from NITE Biological Resource Center (Japan). *Escherichia coli* DH5α was used for gene cloning and *E. coli* BL 21 (DE3) was used for protein expression. The plasmid pSMT3 that carries an N-terminal His-SUMO tag (His-tagged small ubiquitin-related modifier) was utilized as an expression vector. *A. ishigakiensis* NBRC 107699^T was grown in Marine Broth 2216 (BD Difco^{TM}, USA). The cloning and expression strains were both grown at 37 °C in Luria–Bertani (LB) medium (Sangon Biotech, China).

Reagents and chemicals

The *p*-nitrophenyl (*p*-NP) esters including *p*-NP acetate (*p*-NPC2), *p*-NP butyrate (*p*-NPC4), *p*-NP caprylate (*p*-NPC8), *p*-NP decanoate (*p*-NPC10), *p*-NP laurate (*p*-NPC12), *p*-NP myristate (*p*-NPC14) and *p*-NP palmitate (*p*-NPC16) were purchased from Sigma-Aldrich (USA), and *p*-nitrophenyl hexanoate (*p*-NPC6) was purchased from TCI (Japan). Acyl-CoA thioesters including hexanoyl-CoA (C6-CoA), octanoyl-CoA (C8-CoA), decanoyl-CoA (C10-CoA), lauroyl-CoA (C12-CoA), myristoyl-CoA (C14-CoA), palmitoyl-CoA (C16-CoA), and stearoyl-CoA (C18-CoA), and 5,5′-dithiobis (2-nitrobenzoate) (DTNB) were purchased from Sigma-Aldrich (USA). All other reagents used were of analytical grade.

Sequence and phylogenetic analysis

The putative esterase gene *ali5* was screened and identified from the annotated data of the genome of *A. ishigakiensis* NBRC 107699^T, which was sequenced previously (data not shown). Homologous amino acid sequences of the deduced amino acid sequence of Ali5

were searched and analyzed using BLASTp programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using Clustal X version 2.0, and the results were rendered using the ESPript version 3.0 online tool (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The theoretical molecular mass (MW) and isoelectric point (pI) of the esterase were computed via the ExPASy website (http://www.expasy.org/proteomics). The evolutionary relationship and corresponding phylogenetic tree were determined and constructed using the neighbor-joining method with MEGA version 5.0 software.

Cloning, expression, and purification

The complete genome of *A. ishigakiensis* NBRC 107699^T, which contains the gene *ali*5, was extracted by using a Bacterial Genomic DNA Miniprep Kit (Axygen, USA). The esterase gene *ali*5 was amplified by primer pairs (Table 1) with primer STAR GXL DNA polymerase (TaKaRa, China) via PCR. The amplicon was cloned into pSMT3 expression and the vector was then transformed into *E. coli* BL21 (DE3) for protein expression.

Escherichia coli BL21 (DE3) strains were cultivated in LB medium with 50 μg/ml of kanamycin at 37 °C and 200 rpm until the OD_{600} reached 0.6–0.8. Then isopropyl β-D-1-thiogalactoside (IPTG) was added at a final concentration of 0.5 mM to induce protein expression within 20 h at 16 °C. The cells were collected by centrifugation at 12000×g for 10 min and washed twice with PBS buffer (0.8% NaCl, 0.02% KCl, 0.142% Na_2HPO_4, and 0.027% KH_2PO_4, pH 7.4), then resuspended and transferred into start buffer (50 mM Tris/HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0). After ultrasonic disruption for 30 min (work 2 s/intermittent 3 s, 350 W) on ice, the fragments were removed by centrifugation at 18,000×*g*, 4 °C for 30 min. Supernatants containing the fusion protein were incubated with a Ni–NTA agarose column for affinity purification. The fusion protein can be combined with the Ni–NTA agarose column and 20 mM imidazole in the start buffer was used to prevent non-specific binding of other protein. Then, the elution buffer (50 mM Tris/HCl, 500 mM NaCl, 250 mM imidazole, pH 8.0) was used to elute the fusion protein. By adding the ubiquitin-like specific protease 1 (ULP1) to the protein eluate, the His-tagged SUMO was cleavaged from the fusion protein after overnight digestion at 4 °C. The semi-permeable membrane was used to dialyze the 250 mM of the imidazole in the protein eluate to 20 mM. The digested products were then passed through the Ni–NTA agarose column again to capture and remove the His-tagged SUMO. The expression and purities of the protein were verified by SDS-PAGE (12% polyacrylamide gels).

Mutagenesis

The point mutants Asn55Asp (N55D) and Tyr196Gly (Y196G) were generated by site-directed mutagenesis using wild-type recombinant plasmid as the template with the *Fast* Mutagenesis system (Transgene Biotech, China) via whole-plasmid PCR in 18 reaction cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min. After verification by DNA sequencing, the mutant recombinant plasmids were transformed into *E. coli* BL21 (DE3) for protein expression.

Enzyme activity assay

Esterase activity was evaluated by measuring the UV absorption (405 nm) at 40 °C over 2 min. In detail, the standard reaction mixture (1 ml) contained 1 mM of *p*-NPC4 (dissolved in acetonitrile), 1–1.5 μg of purified esterase, and 100 mM of phosphate buffer (pH 7.5). The enzyme activities were calculated by use of the molar extinction coefficient of *p*-nitrophenolate (ε = 14150 M^{-1} cm^{-1}). Thioesterase activity was

Table 1 Primers used in this study

Ali5	Forward	5′-CGCGGATCCATGCAAAAAGGCGTTTGGTCGATCA-3′
	Reverse	5′-CCGCTCGAGTTAGCTTTGCTCTTCAGGAAGCGCG-3′
(N55D)	Forward	5′-ATATACTGGCTTTTGGTGACAGTCTGTTCGCCGGGTA-3′
	Reverse	5′-TACCCGGCGAACAGACTGTCACCAAAAGCCAGTATAT-3′
(Y196G)	Forward	5′-TGGCTTGAATCTATCGGCCAAGACC CGACGCTG-3′
	Reverse	5′-CAGCGTCGGGTCTTGGCCGATAGATTCAAGCCA -3′

*Bam*HI/*Xho*I restriction sites and modified codons are underlined

determined by measuring the UV absorption (412 nm) at 35 °C within 4 min. The reaction mixture contained 25 μM of C16-CoA (dissolved in acetonitrile), 0.5 mM of DTNB, 1–1.5 μg of purified esterase, and Tris/HCl buffer (100 mM, pH 7.5) in a final volume of 1 mL. The enzyme activities were calculated by use of the molar extinction coefficient (ε = 13600 M^{-1} cm^{-1}). One unit of enzyme activity (1 U) was defined as the amount of enzyme required to catalyze the decomposition of substrates to release 1 μmol of (fatty) acid per minute (Lescic Asler et al. 2010).

Enzyme characterization

The esterase and thioesterase substrates specificities of Ali5 were ascertained by using *p*-NP esters with different carbon chain lengths (C2–C16) and acyl-CoA thioesters with different chain lengths (C6–C18) with the standard measurement method described above. The optimum temperature was determined by evaluating the enzyme activity over a range of 10–60 °C (intervals of 5 °C). The optimum pH assay was performed at 348 nm, the pH-independent isosbestic wavelength of *p*-nitrophenol and *p*-nitrophenolate, by using overlapping buffer systems. To evaluate thermostability, an equal amount of enzyme was incubated at temperatures in the range of 10–70 °C (intervals of 5 °C) for 1 h and 2 h, respectively. Residual activity was determined at the optimum reaction conditions determined above. The effects of the divalent metal ions and chelator EDTA were determined at a final concentration of 10 mM. The effect of NaCl was determined at final concentrations of 0.5 M, 1 M, 2 M, 3 M, 4 M and 5 M. The effects of organic solvents were determined at final concentrations of 5% and 15% (v/v). The effects of detergents were determined at final concentrations of 1% and 5% (v/v). The test reagents were added to the reaction buffer and incubated to designated temperature then the enzyme and substrate were added for measurement.

The kinetic parameters of esterase activity were obtained by measuring the initial reaction rate of the enzyme using *p*-NPC4 as a substrate at various concentrations (0.1–3.0 mM) at 40 °C for 2 min, and the V_{max} and K_m were calculated by a Linweaver–Burk plot using Michaelis–Menten equation with Graphpad Prism software. Effects of substrate concentrations on thioesterase activity of enzymes were measured by using C16-CoA as a substrate at various concentrations (2.5–100 μM) at 35 °C within 4 min.

Each set of experiments contained triplicate reactions and a control containing thermally inactivated enzyme. Data were presented as mean ± SD. Statistical analyses were performed with Student's *t*-tests. P values less than 0.05 were considered statistically significant.

Structural modeling and putative structure analysis

The three-dimensional (3D) structure of Ali5 was built using the SWISS-MODEL server (https://swissmodel.expasy.org/). The predicted structural model figures were generated and analyzed by PyMoL software (http://pymol.sourceforge.net), and the surface electrostatic potential was visualized with the assistance of the APBS plugin.

Nucleotide and amino acid sequences accession numbers

The genome sequence of *A. ishigakiensis* NBRC 107699^T has been deposited in the GenBank database under Accession Number CP015963. The locus tag of the *ali5* gene is A6F69_RS05635, and the amino acid sequence accession number of Ali5 is WP06758468.

Results

Sequence and phylogenetic analysis of Ali5

By analyzing the annotated genomic data of *A. ishigakiensis* NBRC 107699^T, a putative gene, *ali5*, with a length of 705 nucleotides encoding an esterase, Ali5, with 234 amino acid residues was identified. The MW and pI of the esterase calculated by the ExPASy website were 25.181 kDa and 4.33, respectively.

Searches of available homologous amino acid sequences in the GenBank nr database and Protein Data Bank (PDB) database showed that Ali5 shares high sequence identities (> 50%) with putative arylesterases that have not been characterized. The phylogenetic tree of Ali5 and the eighteen known bacterial lipolytic enzyme families which have been identified was constructed and the results showed that

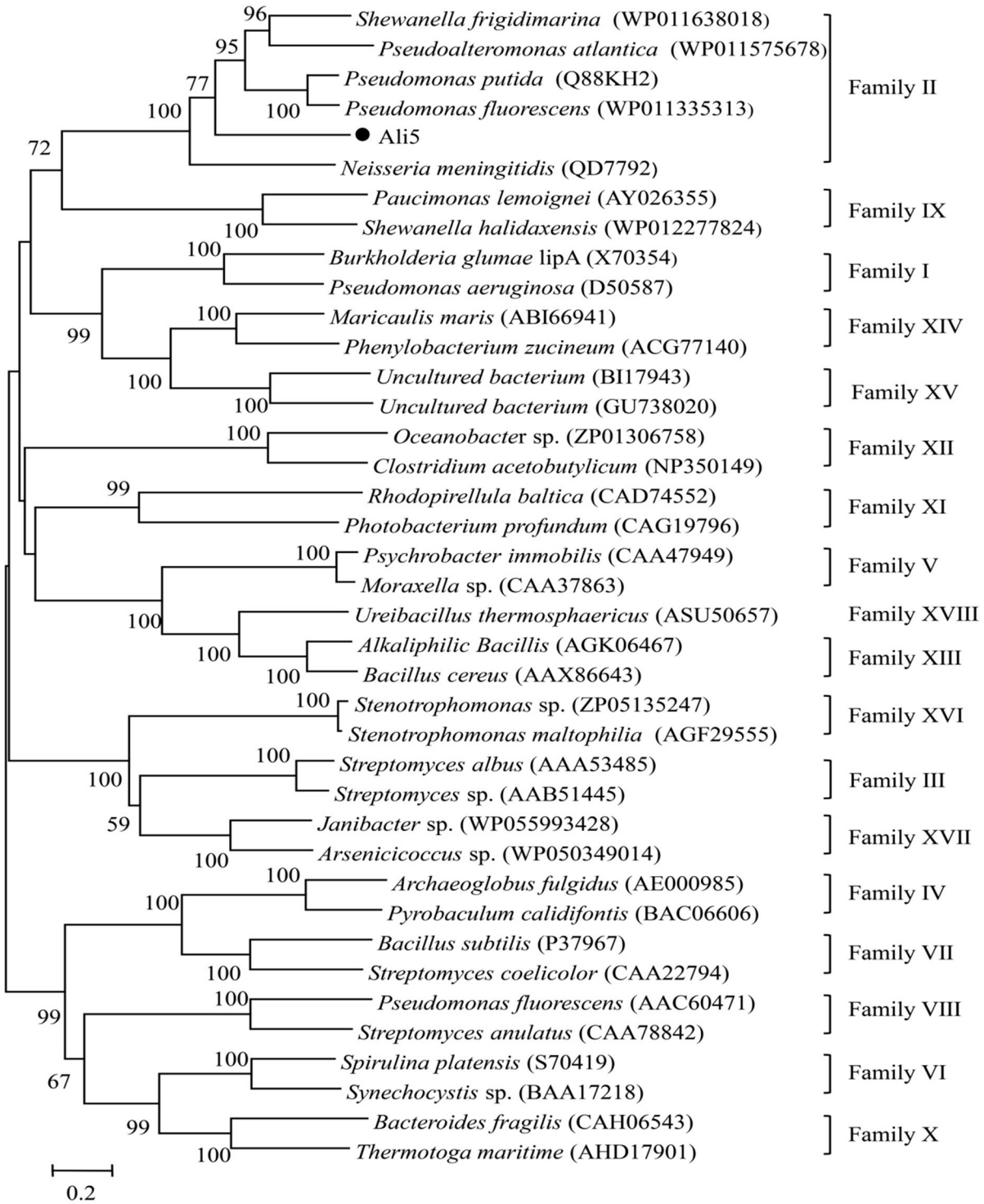

Fig. 1 Phylogenetic analysis of Ali5 and related bacterial lipolytic enzymes was performed using the neighbor-joining method, and the tree was constructed using MEGA version 5.0 software. Bootstrap values are based on 1000 replicates, and only values > 50% are shown. The scale bar indicates the number of amino acid substitutions per site

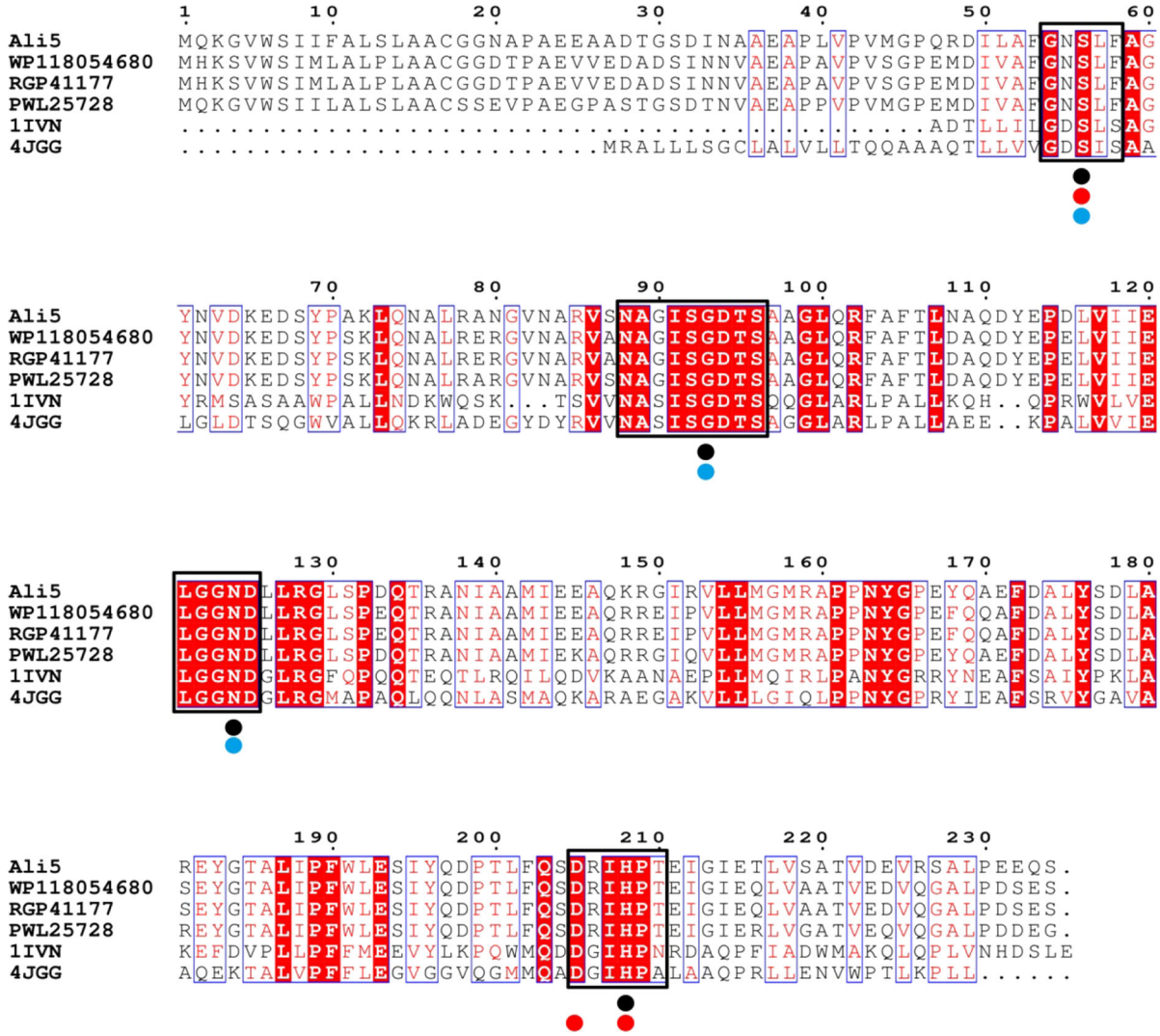

Fig. 2 Sequence alignment analysis of Ali5 with related SGNH family hydrolases. Sequence alignment was performed using ClustalX and rendered using ESPript programs. Accession numbers and PDB codes of the enzymes in the NCBI database are given for Ali5 (from this study), PWL25728 (*Alterery-throbacter* sp. XM-24bin4), RGP41177 (*Altererythrobacter* sp. BPTF-M16), 1IVN (*E. coli*) and 4JGG (*P. aeruginosa*). Identical and similar residues among groups are annotated in whitetext on a red background and in red text on a white background, respectively. Four conserved sequence blocks (I, II, III and IV) and the characteristic residues contained in each conserved block are indicated in black boxes and black dots, respectively. The constituent residues of the catalytic triad and the oxyanion hole are indicated by red and blue dots, respectively

Ali5 clustered into a branch belonging to the family II (Fig. 1).

Multiple sequence alignment of Ali5 with highly related homologs identified four conserved blocks (I, II, III and V) containing specific residues (Ser^{56}, Gly^{93}, Asn^{124} and His^{208}) of SGNH family hydrolases in the amino acid sequence of Ali5. The critical residues that participate in the formation of the GNSL motif, catalytic triad, oxyanion hole with Ser^{56} were arranged at specific positions in the corresponding blocks (Fig. 2). The multiple sequence alignment and phylogenetic analysis results above confirmed that Ali5 is a novel SGNH family hydrolase.

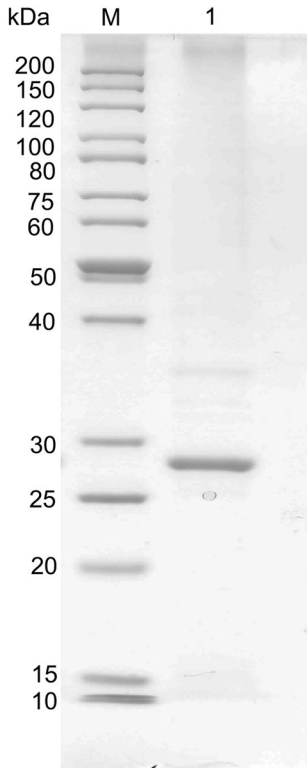

Fig. 3 SDS-PAGE analysis of SUMO tag-based purified Ali5. Lane M, Standard protein molecular mass marker; Lane 1, purified recombinant protein

Expression and purification

The recombinant plasmid containing the correct gene fragment coding for Ali5 was transformed into *E. coli* BL21 (DE3) for overexpression. After induction by IPTG at 20 °C for 20 h, the Ali5 protein was affinity-purified using a Ni–NTA agarose column. The SUMO-tag was cleaved from fusion protein by added the ULP1 protease to the protein eluate. Then the products were passed through the Ni–NTA agarose column again to capture the His-tagged SUMO, and the purified protein was obtained. The MW of Ali5 was approximately 27 kDa as determined by SDS-PAGE analysis, which was close to the calculated value (Fig. 3).

Esterase activity and substrate specificity of Ali5

The esterase substrate specificity of Ali5 was measured by using *p*-NP esters with various acyl chain lengths (C2–C16) (Fig. 4a). Ali5 exhibited high hydrolysis activity on short-chain (C2–C10) *p*-NP esters, with the maximum esterase activity toward *p*-NPC4 (14.6 U/mg). The kinetic parameters were determined by using *p*-NPC4 was used as the substrate, and the results are given (Table 2, Supplementary Fig. 1).

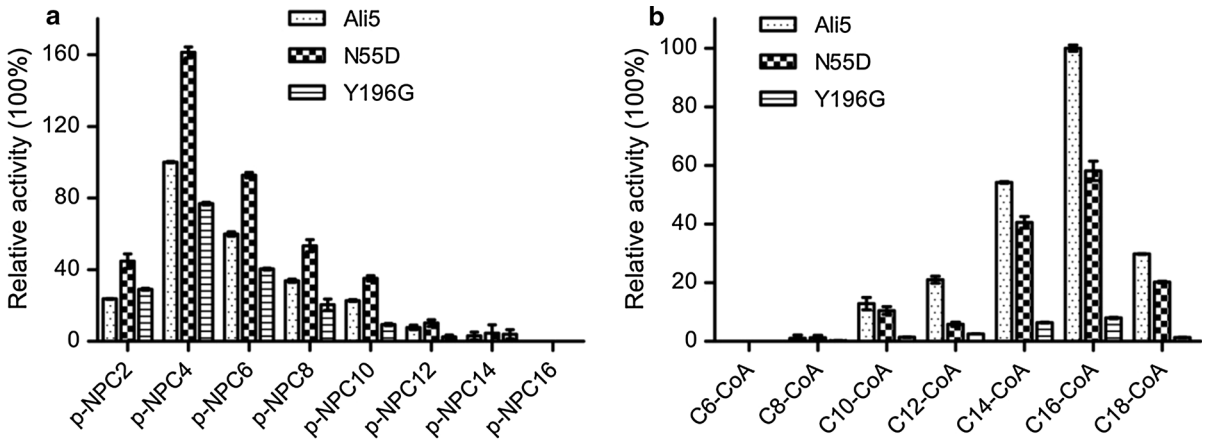

Fig. 4 Substrate specificity of Ali5, N55D and Y196G toward *p*-NP esters and acyl-CoA thioesters. **a** Esterase substrate specificity was determined using *p*-NP esters with various chain lengths (C2–C16) at a final concentration of 1 mM at 40 °C in phosphate buffer (pH 7.5). **b** Thioesterase substrate specificity was determined using acyl-CoA thioesters with different chain lengths (C6–C18) at 35 °C in Tris/HCl buffer (pH 7.5). The highest enzyme activity was taken as 100%. The data are shown as mean ± SD (n = 3)

Table 2 Kinetic parameters of Ali5, Ali5-N55D and Ali5-Y196G

Substrates	Enzyme	V_{max} [μmol/mg/min]	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}\mu M^{-1}$]
p-NPC4	Ali5	30.2 ± 0.9	559.5 ± 0.05	12.66 ± 0.39	0.023
	Ali5-N55D	34.0 ± 0.9	611.2 ± 0.04	14.26 ± 0.37	0.023
	Ali5-Y196G	10.8 ± 0.2	149.4 ± 0.01	4.52 ± 0.07	0.030

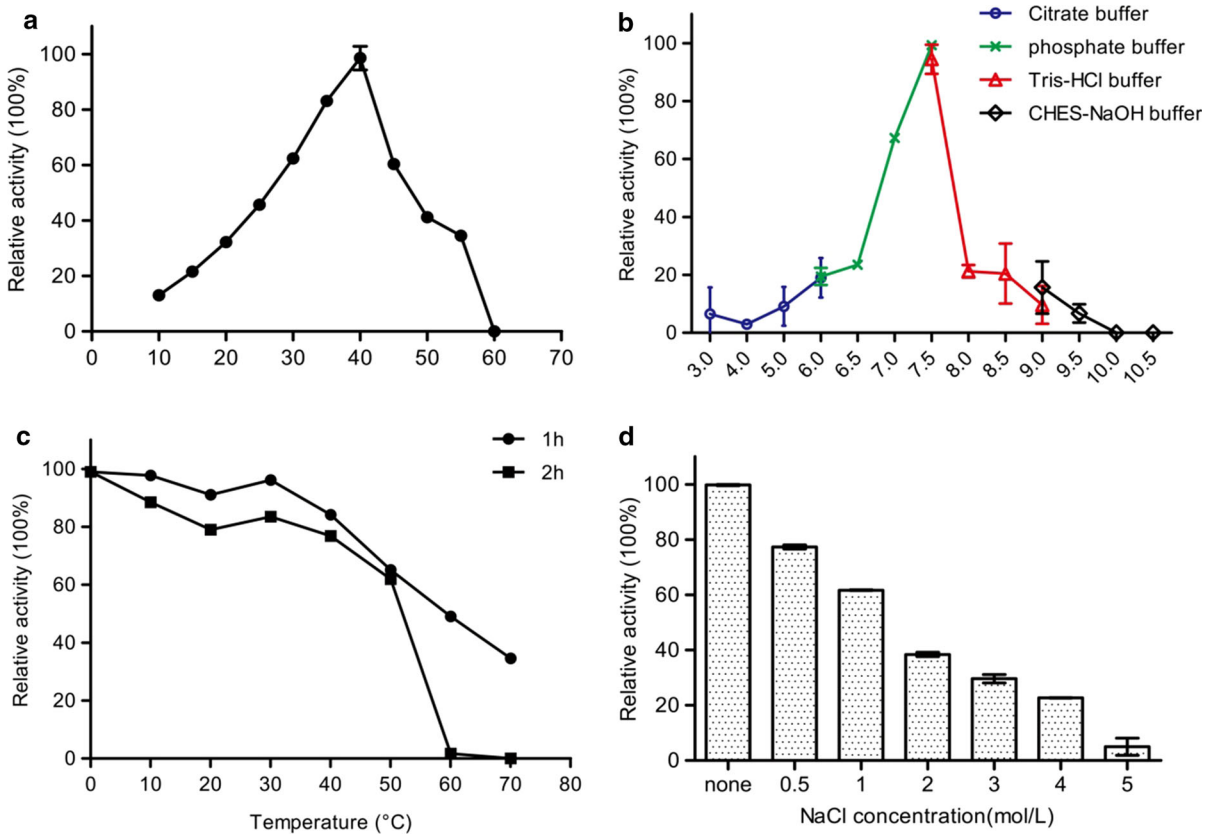

Fig. 5 Effect of temperature (**a**), pH (**b**), thermostability (**c**) and NaCl (**d**) on the activity of Ali5. **a** The catalytic activity was determined using *p*-NP butyrate (*p*-NPC4) in phosphate buffer (pH 7.5). **b** The catalytic activity was determined using *p*-NPC4 at 40 °C in buffer with different pH values: 100 mM citrate buffer (pH 3.0–6.0), 100 mM phosphate buffer (pH 6.0 to 7.5), 100 mM Tris/HCl buffer (pH 7.5 to 9.0) and 50 mM CHES-NaOH buffer (pH 9.0–10.5), at a final concentration of 100 mM. **c** The residual activity was determined at 40 °C in phosphate pH 7.5 using *p*-NPC4 as a substrate after incubation at 10–70 °C (intervals of 5 °C) for 1 h and 2 h, respectively. **d** The residual activity was determined at 40 °C using *p*-NPC4 as a substrate in phosphate pH 7.5 containing NaCl at final concentrations of 0 M, 0.5 M, 1 M, 2 M, 3 M, 4 M and 5 M in the standard reaction mixture. The highest enzyme activity was taken as 100%. The data are shown as mean ± SD (n = 3)

Thioesterase activity and substrate specificity of Ali5

The thioesterase substrates specificity of Ali5 was measured by using acyl-CoA thioesters with various acyl chain lengths (C6–C18) (Fig. 4b). Ali5 exhibited hydrolysis activity for long-chain (> 8) acyl-CoA thioesters, with the maximum activity toward C16-CoA (4.2 U/mg). The effect of substrate concentration on thioesterase activity of Ali5 was determined by using C16-CoA as the substrate and the results are given in supplementary Fig. 2.

Table 3 Effects of various metal ions and the chelator EDTA on the enzyme activity of Ali5

Metal/chelating agent (10 mM)	Relative activity (%)
Control	99.3 ± 0.3
Ni^{2+}*	49.3 ± 0.1
Co^{2+}*	80.2 ± 0.2
Sr^{2+}*	109.5 ± 0.3
Mg^{2+}*	108.2 ± 0.3
Ca^{2+}*	86.4 ± 0.7
Mn^{2+}*	118.1 ± 1.5
Cu^{2+}*	3.7 ± 1.0
Ba^{2+}*	110.2 ± 0.8
Zn^{2+}*	37.6 ± 2.7
Cd^{2+}*	94.1 ± 0.9
EDTA*	136.4 ± 1.8

Activities were determined using *p*-NP butyrate as the substrate at 40 °C and pH 7.5. The value observed without metal ion or chelator was taken as 100%

*$p < 0.05$, representing a significant difference from the control (Student's *t* test)

Activities and substrate specificities of N55D

The mutant N55D was constructed to explore the effect of residue substitution (Asn^{55} to Asp) in the GNSL motif on catalytic activity and substrate specificities. The esterase substrate specificities of N55D were almost unchanged, and the esterase activities towards *p*-NP esters were increased, with the maximum esterase activity toward *p*-NPC4 was increased from 14.6 U/mg to 23.6 U/mg (Fig. 4a). In addition, in terms of thioesterase substrate specificity, the catalytic activity of N55D was decreased. N55D still exhibited the maximum thioesterase activity toward CoA-C16, although the level of activity was decreased (from 4.2 U/mg to 2.4 U/mg) (Fig. 4b).

Enzyme characterization

The optimum reaction conditions of Ali5 were determined by measuring the catalytic activities over pH and temperature ranges of 3.0–10.5 and 10–60 °C with *p*-NPC4 as the substrate. Ali5 exhibited a narrow pH and temperature range, with the highest activity at 40 °C (Fig. 5a) in phosphate buffer pH 7.5 (Fig. 5b). Approximately 40% of activity was maintained when 2 mol/L NaCl was present in the reaction mixture, indicating that Ali5 is a salt-tolerant enzyme (Fig. 5c). Ali5 also performed well in terms of thermostability, showing more than 60% of enzyme activity was retained when incubated at 50 °C for 2 h, and 50% of activity was retained after incubation at 60 °C for 1 h (Fig. 5d).

The effect of divalent metal ions and the chelator EDTA on enzyme activity indicated that EDTA could increase the enzyme activity of Ali5 by 30%. Ba^{2+}, Mn^{2+}, Sr^{2+} and Mg^{2+} also promoted the enzyme activity of Ali5. By contrast, Cu^{2+} strongly or almost

Table 4 Effects of various organic solvents and detergents on the activity of Ali5

Solvent types	Relative activity (%)		Detergent types	Relative activity (%)	
	Solvents (5% v/v)	Solvents (15% v/v)		Detergents 1% (v/v)	Detergents 5% (v/v)
Control	100.0 ± 0.5	100.0 ± 1.1	Control	100.0 ± 0.5	100.0 ± 1.1
Isopropanol	83.2 ± 2.7*	n.d.	Tween 20	12.3 ± 0.1*	n.d.
Acetonitrile	52.6 ± 0.4*	n.d.	Tween 80	5.8 ± 1.1*	n.d.
Ethanol	86.0 ± 3.9*	3.0 ± 1.4*	TritonX-100	n.d.	n.d.
Methanol	125.9 ± 0.9*	29.5 ± 0.9*			
Acetone	81.0 ± 0.6*	1.6 ± 0.3*			
DMSO	122.5 ± 2.8*	90.1 ± 2.8*			
Glycerol	163.0 ± 0.4*	176.3 ± 1.2*			
DMF	83.2 ± 0.6*	n.d.			

Activities were determined using *p*-NP butyrate as the substrate at 40 °C and pH 7.5. The value observed without organic solvent or detergent was taken as 100%

*$p < 0.05$, representing a significant difference from the control (Student's *t* test)

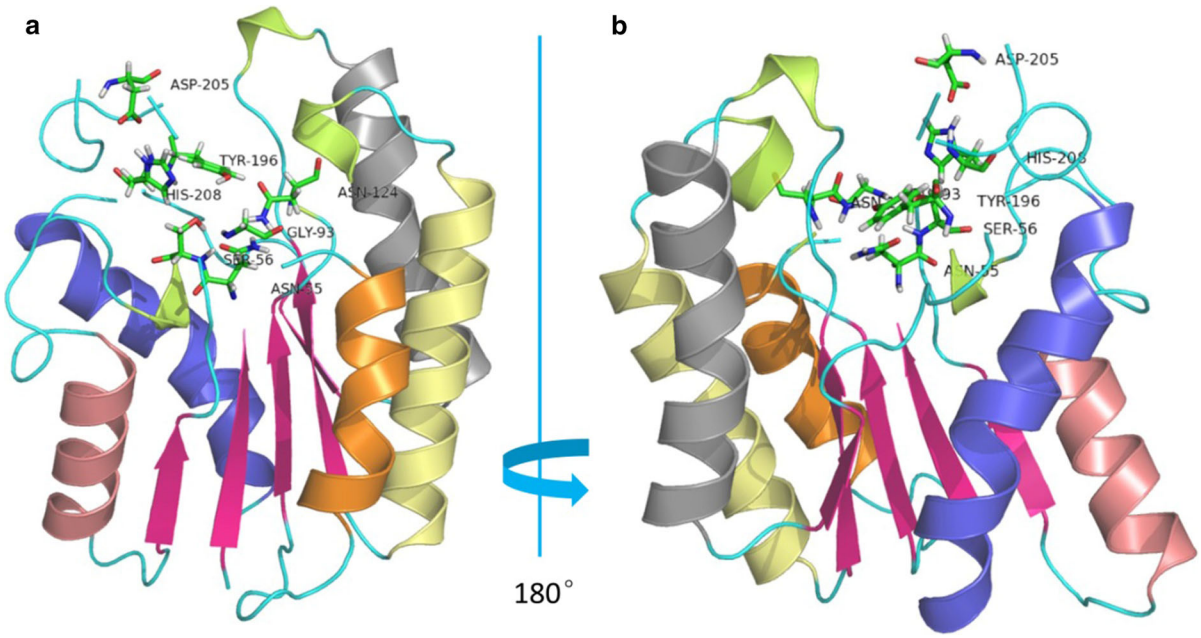

Fig. 6 Homology modeling of the Ali5 Structure. **a** Model constructed by the SWISS-MODEL server with TesA (PDB code: 4JGG) of *P. aeruginosa* as the template. The residues of the catalytic triad are shown as stick models. **b** 180° rotated view of (**a**)

completely inhibited the hydrolysis activities of Ali5 (Table 3).

The tolerance of various organic solvents and detergents showed that Ali5 was active in the presence of low concentrations (5% v/v) of organic solvents and that enzyme activity was promoted by glycerol, methanol and DMSO. However, the enzyme activity was nearly completely inhibited by 15% (v/v) organic solvents except glycerol and DMSO, and glycerol increased the activity by 170%. The tested detergents strongly inhibited the activity of Ali5 and Triton X-100 completely inactivated Ali5 (Table 4).

Structural modeling and putative structure analysis

It was tried to screen protein crystals of Ali5, but unfortunately, no crystal growth was observed. After the amino acid sequence of Ali5 was uploaded, the protein structure TesA (PDB code: 4JGG) of *P. aeruginosa* was selected as the template, the structure model was automatically constructed by the SWISS-MODEL server. The structural model of Ali5 is a classical α/β/α fold containing a five-stranded central parallel β-sheet surrounded by five α helices and three 3_{10} helices (Fig. 6). Furthermore, structural comparisons with TesA and the classic SGNH family hydrolase TAP revealed that Ali5 has a substrate binding cavity similar to that of TesA but different from the substrate binding crevice of TAP (Fig. 7). More importantly, analysis of the structural model of Ali5 indicated that the residueTyr^{196} is located near the substrate binding cavity (Fig. 8). Therefore, we constructed the mutant Y196G and measured its activity to assess the effect of Tyr^{196} on the catalytic activity of Ali5.

Activities and substrate specificities of Y196G

The assay of esterase substrate specificity showed that the activity of Y196G toward *p*-NP esters with acyl chains longer than C4 was decreased. Y196G also exhibited the maximum esterase activity toward *p*-NPC4, although the activity decreased from 14.6 U/mg to 11.2 U/mg (Fig. 4a). Similarly, the thioesterase activity of Y196G was decreased toward acyl-CoA thioesters (Fig. 4b). More importantly, Y196G still exhibited the maximum thioesterase activity toward CoA-C16, but the activity was reduced by more than 90% (from 4.2 U/mg to 0.33 U/mg).

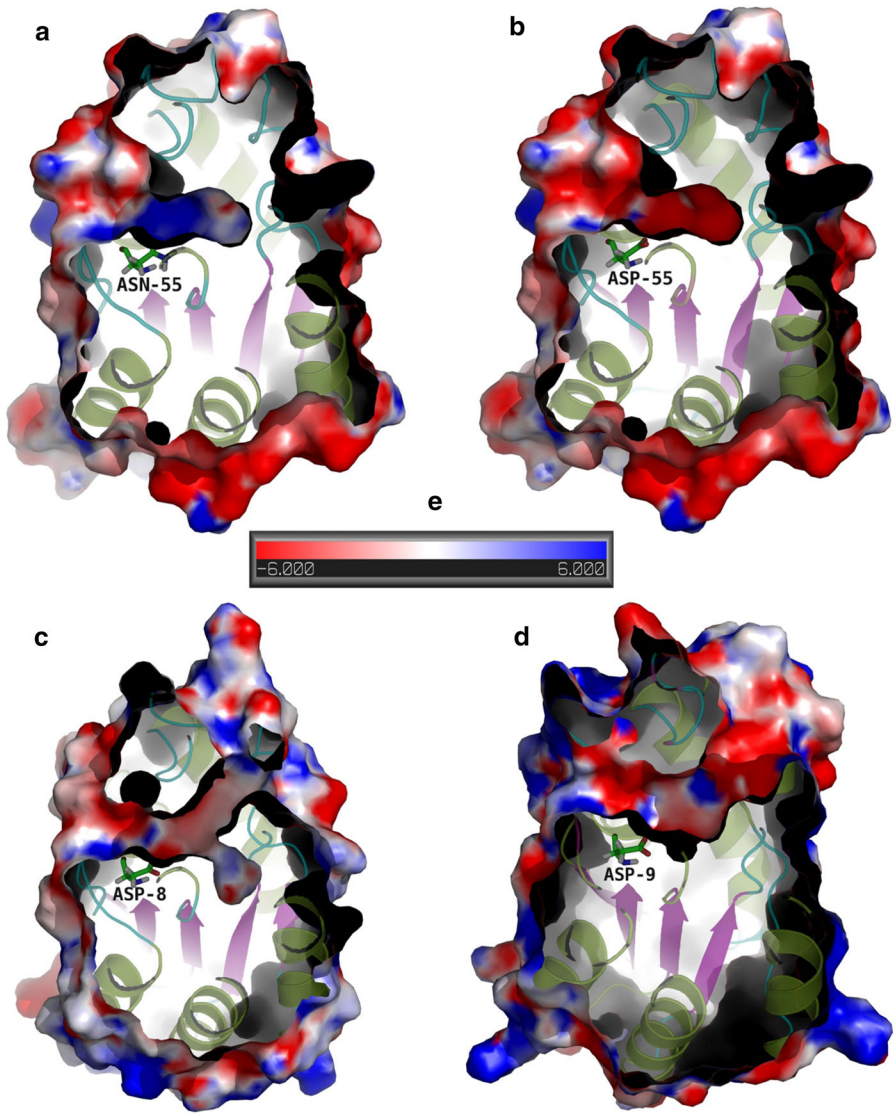

Fig. 7 Surface electrostatic potential analysis of Ali5 and N55D and comparison with TesA and TAP. **a** The surface electrostatic potential of Ali5. **b** The surface electrostatic potential of N55D. **c** The surface electrostatic potential of TesA. **d** The surface electrostatic potential of TAP. **e** Positive and negative electrostatic potentials are indicated by *blue* and *red*, respectively. The surface electrostatic potential was obtained by PyMoL and the APBS plugin. The substrate binding pockets are indicated with a yellow dashed circle, and the residues Asn in the GNSL motif and Asp in the GDSL motif are indicated as stick models

Discussion

The abundant microbial resources of marine environments have become a valuable source of new SGNH family hydrolases. In particular, continuous developments in genome sequencing technologies allow a large number of bacterial genome datasets to be obtained more conveniently and have become an effective means of screening novel biocatalysts. Using this strategy, a novel SGNH family hydrolase Ali5 from the annotated genome of *A. ishigakiensis* NBRC 107699^{T} was identified.

It has previously been reported that the Leu residue in the GDSL motif of the SGNH family hydrolases is not strictly conserved (Arpigny and Jaeger 1999), but the substitutions of the Asp residue in the GDSL motif have not been reported previously. Interestingly, homologous sequences alignment revealed a GNSL motif in the amino acid sequence of Ali5 that differed from the classic GDSL motif. Furthermore, this GNSL motif was found in several putative arylesterases obtained by genomic annotation in *Alphaproteobacteria*, such as an arylesterase (PWL25728) from *Altererythrobacter* sp. XM-24bin4, arylesterases (WP118054680 and RGP41177) from *Altererythrobacter* sp. BPTF-M16, an arylesterase (WP047806778) from *Altererythrobacter marensis*, an arylesterase (WP086436309) from

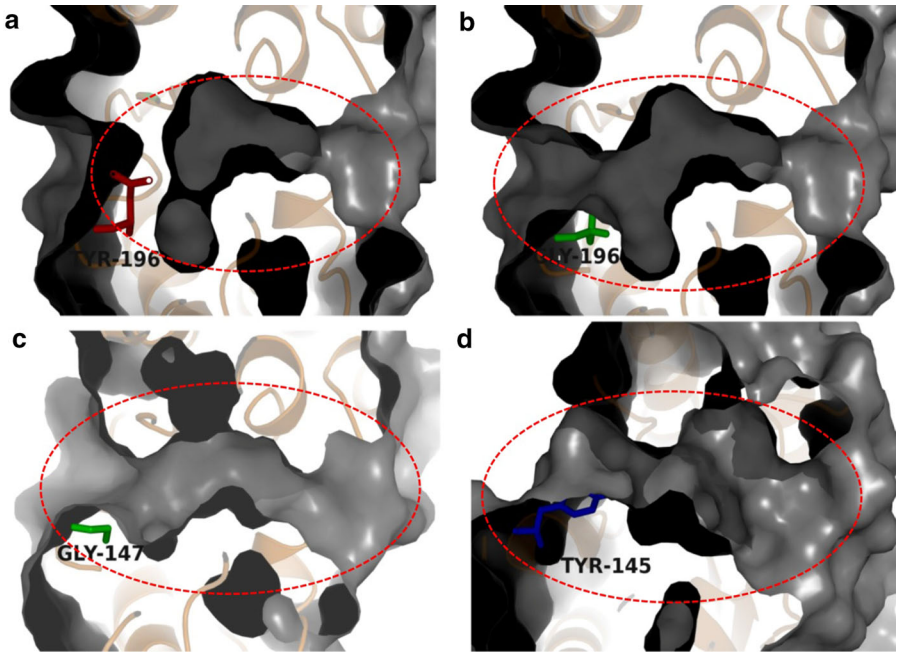

Fig. 8 Substrate binding pockets of Ali5, Y196G, TesA and TAP. Surface view of the structures showing the difference in the substrate binding pockets of Ali5 (**a**), Y196G (**b**), TesA (**c**) and TAP (**d**). The substrate binding pockets are indicated with red dashed circles. The residue affecting the length of the substrate binding pocket of Ali5 and the mutated residue are shown as stick models

Altererythrobacter xiamenensis, an arylesterase (WP115367444) from *Altererythrobacter* sp. HME9302 and an arylesterase (WP067609223) from *Erythrobacter* sp. QSSC1-22B. The homologous sequence alignment results indicated that the substitution of Asp residue with Asn in the GDSL motif is ubiquitous in bacterial SGNH-hydrolases of the family *Erythrobacteraceae*, particularly in bacteria of the genus *Altererythrobacter*, rather than accidental. However, the effect of the substitution of the Asp residue in the GDSL motif on catalytic function has been unclear before.

The purified Ali5 exhibited hydrolysis activity on short-chain (< 10) *p*-NP esters and no significant activity with long-chain (> 10) *p*-NP esters, thus indicating that Ali5 is an esterase rather than a lipase. The thioesterase activity of Ali5 has also been found in some other SGNH family hydrolases like TesA (Kovacic et al. 2013). Interestingly, most of the SGNH family esterases prefer short-chain *p*-NP esters, for SGNH family hydrolase with thioesterase activities, are prefer long-chain acyl-CoA thioesters and most of them showed the highest activities towards palmitoyl-CoA. The narrow pH and temperature range indicated that Ali5 sensitive for a temperature and pH. The optimal temperature and pH are lower than some other SGNH family hydrolases. For example, EstL5 from *Geobacillus thermodenitrificans* T2 (Yang et al. 2013), FNE from *Fervidobacterium nodosum* Rt17-B1 (Yu et al. 2010) and Est19 and Est8 from *Bacillus* sp. K91 (Ding et al. 2014; Yu et al. 2016). In addition, Ali5 lost most of activity at the pH below 6.0 is similar to most of SGNH family hydrolases like (Yu et al. 2016). Ali5 has a lower optimum reaction temperature but performs well in terms of thermostability similar to estSL3 from *Alkalibacterium* sp. SL3 (Wang et al. 2016). In addition, the well performance of Ali5 in terms of salt, metal ions and organic solvent tolerance is also consistent with the characteristics of marine-derived esterases and SGNH family hydrolases.

The negatively charged residue Asp^{9} located in the GDSL motif and near the catalytically active Ser^{10} of the SGNH family hydrolase TAP was considered to facilitate expulsion of the negatively charged reaction product (Lo et al. 2003). Surface electrostatic potential analysis showed that the substrate binding cavity region near the uncharged residue Asn^{55} located in the GNSL motif of Ali5 exhibited a positive (blue) surface electrostatic potential, which changed to negative (red) when this residue was substituted by the negatively charged residue Asp in N55D (Fig. 7a, b). The surface potential of the substrate binding cavity region near the substituted residue Asp55 in N55D was similar to esterase TesA and TAP in that region

(Fig. 7c, d). In addition, a slight decrease in substrate affinity (K_m) and increases in the catalytic activity (V_{max}) and turnover rate (k_{cat}) of N55D toward *p*-NPC4 were observed (Table 2), thus demonstrating that the substitution of Asn^{55} by Asp affects the esterase catalytic activity of Ali5, but not significantly. However, the decreased catalytic activity of N55D toward C16-CoA indicated that Asn^{55} is advantageous for the thioesterase activity of Ali5 (Fig. 4). Furthermore, it is speculated that other residues near the substrate binding cavity may have a greater influence on catalytic activity.

Structural analysis indicated that residue Tyr^{196} may have an effect on the shape of the substrate binding cavity of Ali5. It was found that Ali5 may have a short substrate binding cavity unlike the long substrate binding cavity of TesA (Kovacic et al. 2013) and the substrate binding crevice of TAP (Lo et al. 2003) (Fig. 8). When residue Tyr^{196} was substituted by the less bulky glycine residue, the catalytic activities of Y196G towards *p*-NP esters and acyl-CoA thioesters decreased, indicating that Tyr^{196} may have an effect on the structure stability and catalytic activity of Ali5 (Fig. 4). Since the substrate affinity of Y196G towards *p*-NPC4 was increased, it is speculated that Tyr^{196} may be helpful for the substrate binding to Ali5.

In summary, we identified and characterized a new SGNH family hydrolase, Ali5, which contains a novel GNSL motif that also found in several putative arylesterases that differs from the classic GDSL motif of known SGNH family hydrolases. Moreover, Ali5 has esterase and thioesterase activities and performs well in terms of NaCl, metal ion and organic solvent tolerance and thermostability. Moreover, residue Tyr^{196} in the substrate binding cavity may have an effect on structure and catalytic activity of Ali5. All of these enzymatic properties and structural features make Ali5 a good candidate for research on the catalytic mechanism of SGNH family hydrolases, enzyme engineering and biotechnological applications.

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Supporting information Supplementary Fig. 1—Kinetics plots of Ali5, N55D and Y196G for substrates *p*-NP butyrate (*p*-NPC4). **a** Michaelis–Menten plot of Ali5 for *p*-NPC4. **b** Michaelis–Menten plot of N55D for *p*-NPC4. **c** Michaelis–Menten plot of Y196G for *p*-NPC4. All values are the average of three independent assays.

Supplementary Fig. 2—Effects of substrate concentrations on thioesterase activity of Ali5, N55D and Y196G, with palmitoyl-CoA (C16-CoA) as the substrate. **a** Effects of C16-CoA concentrations on thioesterase activity of Ali5. **b** Effects of C16-CoA concentrations on thioesterase activity of N55D. **c** Effects of C16-CoA) concentrations on thioesterase activity of Y196G. All values are the average of three independent assays.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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