Electronic Supplementary Material (ESI) for RSC Chemical Biology. This journal is © The Royal Society of Chemistry 2021

**Electronic Supplementary Information** 

# Insights into Phosphatase-activated Chemical Defense in a Marine Sponge Holobiont

Takahiro Jomori, Kenichi Matsuda, Yoko Egami, Ikuro Abe, Akira Takai, Toshiyuki Wakimoto\*

\*Corresponding author. Email: wakimoto@pharm.hokudai.ac.jp

This PDF file includes:

Experimental

Supplementary Figures S1 to S13

Supplementary Tables S1 to S4

Supplementary references

### Experimental

### Assay of pNPP phosphatase activities

The protein phosphatase activities were measured using *p*-nitrophenyl phosphate (*p*NPP) as the substrate, essentially by the method described previously<sup>[1]</sup>. Before use, 5 mM *p*NPP (disodium salt) and 4 mM DL-dithiothreitol (DTT) were dissolved in the buffer solution (40 mM Tris-base, 34 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 4 mM EDTA·2Na without pH adjustment). The reaction was started by the addition of 190  $\mu$ L of reaction buffer to the well containing 10  $\mu$ L of the fractionated enzyme solution, and the initial rate of *p*-nitrophenol liberation was measured by recording the change in the absorbance at 405 nm with a microplate reader (Thermo Fisher, Labsystems Multiskan MS). All assays were performed at room temperature.

### Assay of phosphocalyculin phosphatase activity for isolation

Aliquots ( $10 \ \mu$ L) of the enzyme fractions were added into the wells of a 96 well-plate containing 6  $\mu$ g of phosphocalyculin A as a substrate, purified by the method described previously<sup>[2]</sup>, or without substrates for background subtraction. After an incubation for 15 min at room temperature, 70  $\mu$ L of double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O) and 20  $\mu$ L of malachite green detection buffer in the kit (BioAssay System, Gentaur) were added to the reaction mixture to detect the hydrolyzed free phosphoric acid. The absorbance at 620 nm was measured with a microplate reader. The relative activity values were obtained by subtracting the values with and without substrates.

### Purification of native phosphocalyculin phosphatase

The marine sponge D. calyx was collected in 2018 by hand, at a depth of 10–20 m, during scuba diving at Izu Peninsula, Japan. The frozen specimens (1.8 kg wet weight) were cut into pieces, homogenized in 3.0 L of 25 mM triethanolamine-HCl buffer, pH 7.5, containing 0.1 mM EGTA, 0.03% (v/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol (buffer A) until a paste form appeared, sonicated for 5 min (at 30-second intervals), and then left on ice for 20 min to allow the sponge debris to settle out. The extract was centrifuged at 7,000  $\times$  g for 20 min at 4 °C, to remove the sponge debris. The supernatant was dialyzed overnight at 4 °C against cold water for desalination. All native phosphatase purifications were performed with an AKTA chromatography system (Pharmacia Biotech, PUMP-50, detection: Control Unit & Optical Unit UV-1 280 nm, collection: GradiFrac) in a refrigerator (SANYO) at 4 °C, except for the last purification step. The crude enzyme solution (7.3 g) was filtered through a 0.45 mm filter and loaded onto two tandemly connected HiPrep 16/10 Q XL (Pharmacia) anion-exchange columns equilibrated with buffer A, at 4 °C. The flow-through fraction of the Q XL columns was subsequently applied to two tandemly connected HiPrep 16/10 SP XL (Pharmacia) cationexchange columns equilibrated in buffer A, and fractionated using a linear gradient from 0 to 250 mM NaCl in buffer A. The fractions (5 mL each) were collected at a flow rate of 5 mL/min. The active fractions were pooled and concentrated using an Amicon 10,000 MWCO centrifugal filter (Merck Millipore) and exchanged to 1.5 M ammonium sulfate in buffer A. The active fraction was loaded onto a 5 mL HiTrap Phenyl Sepharose HP (Pharmacia) hydrophobic interaction column, equilibrated in buffer A with 1.5 M ammonium sulfate. The column was eluted with a 40 mL linear gradient from 1.5 M to 0 mM ammonium sulfate in buffer A, and fractions (2 mL each) were collected at a flow rate of 2 mL/min. The buffer of the active fraction was exchanged to 200 mM NaCl in buffer A and then loaded on a 16/60 Sephacryl S200 (Pharmacia) gel filtration column. The column was eluted with 200 mM NaCl in buffer A, and fractions (1 mL each) were collected at a flow rate of 0.3 mL/min. After desalination, the active fraction was applied to a Mono S 5/50 GL (GE Healthcare) column equilibrated with buffer A, and fractionated in a linear gradient mode from 0 to 250 mM NaCl in buffer A. Fractions (0.5 mL each) were collected at a flow rate of 1 mL/min. The buffer of the active fraction was exchanged to 250 mM NaCl in 50 mM phosphate buffer (pH 7.0). The active fraction was then purified on a PROTEIN LW-803 (Shodex) gel-filtration column with diode array-detected (DAD)-HPLC (SHIMADZU) at a 1.0 mL/min flow rate. The retention time of the active fraction was 10.7 min (Fig. 2A)

### 2D electrophoresis of proteins

The 2D electrophoresis of the purified samples of native phosphatase or recombinant CalL was performed with IPGphor and Multiphor II and an EPS 3501 XL (Pharmacia) electrophoresis power supply, according to the manufacturer's procedure (Pharmacia). The desalted protein solution was mixed with sample buffer (9.8 M urea, 2% [v/v] CHAPS, a trace amount of bromophenol blue, and IPG Buffer pH 3–10) and subjected to isoelectric focusing on an IPG gel, Immobline™ DryStrip gel pH 3–10, 7 cm (GE Healthcare) under a linear gradient of pH 3–10 in 7 cm, which was further developed by SDS-PAGE (ExcelGel SDS Gradient 8–18, 2-D Homogenous 12.5). The proteins in the gel were visualized by silver staining with a Hoefer<sup>®</sup> (Pharmacia) automated gel stainer.

#### Peptide mass fingerprinting for identification of protein

In-gel protein reduction, acetylation and enzymatic digestion were performed by following the reported protocol, with small modifications<sup>[3]</sup>. The active fraction was separated by 10% SDS-PAGE and the proteins were stained with Coomassie Brilliant Blue (CBB, Nacalai Tesque, Fig. 2B). The band containing the protein of interest was excised from the acrylamide gel and cut into pieces in a 1.5 mL tube. The gel pieces were washed three times with 100  $\mu$ L of 25 mM ammonium bicarbonate in 30% CH<sub>3</sub>CN ag., dehydrated by the addition of 100% CH<sub>3</sub>CN, and dried at 56 °C for 5 min. The shrunk gel pieces were swollen in 25 µL of reduction buffer (1 M DTT aq.) at 56 °C for 45 min, and then treated with 100 µL of alkylation buffer (10 mg/mL iodoacetamide aq.) for 30 min at room temperature in the dark. The gel pieces were washed seven times with 100  $\mu$ L of 25 mM ammonium bicarbonate aq., dehydrated by the addition of 100% CH<sub>3</sub>CN, and dried at 56 °C. The shrunk gel pieces were swollen in 25  $\mu$ L of digestion buffer containing 200  $\mu$ g trypsin (Promega V5280, Trypsin Gold, Mass Spectrometry Grade) in 25 mM ammonium bicarbonate aq. on ice. After the gel pieces were incubated at 37 °C overnight, the peptides were extracted twice from the gel with 50 µL of extraction buffer (5% trifluoroacetic acid in 50% CH<sub>3</sub>CN aq.). The concentrated peptides were loaded onto a Pierce C-18 Spin Column (Thermo Scientific) and purified according to the manufacturer's instructions. To improve the amino acid sequence coverage, the remaining gel pieces containing longer peptide fragments were dried and then swollen in 20 µL of second digestion buffer, containing 50 µg endoproteinase Glu-C (Sigma-Aldrich) in 100 mM phosphate buffer on ice. The procedures for the digestion and extraction of peptides were the same as described above. LC-MS/MS analyses were performed with an amaZon SL-NPC (Bruker) operated in the positive mode, coupled with an HPLC system (SHIMADZU). The separation was accomplished by RP HPLC (COSMOSIL 5C<sub>18</sub>-AR-II 4.6 mm I.D. × 100 mm, Nacalai Tesque). Mobile phases A and B were H<sub>2</sub>O + 0.05% trifluoroacetic acid and CH<sub>3</sub>CN + 0.05% trifluoroacetic acid, respectively. Samples were eluted in the gradient mode: 2 to 100% for mobile phase B in 50 min, at a flow rate of 0.2 mL/min. The peptide fragments were detected using the BioTools and Sequence Editor software (Bruker) with the following conditions: enzyme, trypsin or double digestion with trypsin and Glu-C phosphate buffer; modification, carbamidomethyl [Cys] and oxidation [Met]; MS error, ±0.5 Da; MSMS error, ±0.2 Da; Ion mode, positive.

#### Cloning and site-directed mutagenesis of CalL

To construct an expression plasmid for recombinant CalL with a Strep tag II (WSHPQFEK) fused at its C-terminus, the calL gene was amplified with 0.3 mM specific primers (NL\_CalL\_Eco\_F2/CalL-St-HillI\_Re, Table S2), using DNA polymerase 1U KOD One<sup>®</sup> PCR Master mix -Blue- (TOYOBO) and the isolated D. calyx metagenomic DNA fosmid as the template<sup>[2]</sup>. Reactions were run using the following conditions [95 °C 2 min, (98 °C 10 sec, 62 °C 5 sec, 68 °C 5 sec) for 40 cycles, followed by an elongation step at 68 °C 7 min]. The EcoRI/HindIII digested amplicon was inserted into the pUC19 vector (Takara) and transformed into E. coli DH5a chemically competent cells. The plasmid pUC19-calL-strep was extracted and the sequence was confirmed. The plasmid was double digested with Ndel/HindIII and ligated with the pET-22b(+) expression vector (Novagen), and then transformed into E. coli BL21(DE3) chemically competent cells. To introduce mutations of the seven metal-binding residues (I: D116A, Y168F, Y168A, H352A, II: N215A, H305A, H352A, I and II: D165A in Fig. 3) in CalL-strep, all mutated linear plasmids (pUC19-calL-strep) were obtained by PCR of 50 µL reaction mixtures, composed of 25 µL 10× KOD One® PCR Master mix -Blue-, 1.5 µL of 10 mM primers (Table S2), 1 ng of pUC19-calL-strep, and ddH<sub>2</sub>O up to the final 50 µL volume. The PCR was performed under the following conditions [95 °C 2 min, (98 °C 10 sec, 62 °C 5 sec, 68 °C 20 sec) for 18 cycles followed by an elongation step at 68 °C 7 min]. The reaction mixture was processed with Dpnl in 1× CutSmart buffer (New England Biolabs) in a 20 mL reaction volume, to degrade the wild-type calL plasmid. After an incubation at 37 °C for 30 min, the digestion mixtures were fractionated by 1% agarose gel electrophoresis, and the amplicons were excised from the gel and extracted using EconoSpin™ la columns (BIO-PHARMA SERVICES). The eluate was mixed with 10 µL of ddH<sub>2</sub>O and transformed into E. coli DH5a chemically competent cells. Mutations in plasmids were confirmed by sequencing. The mutants in pET22b(+)-calL-strep were transformed into BL21(DE3) cells by following the same procedure for the construction of the wild-type calL expression plasmid.

### Expression and purification of recombinant CalL and its mutants

A single colony of *E. coli* BL21 (DE3) harboring the plasmid pET22b(+)-*calL-strep* or its mutants was picked and used to inoculate 10 mL of 2×YT medium with 100  $\mu$ g/ml ampicillin, and the culture was incubated overnight at 37 °C and 220

rpm. The 1% overnight culture was used to inoculate 200 mL of 2×YT medium containing 100 µg/ml ampicillin and cultured until the OD<sub>600</sub> reached 0.6. To induce protein expression, isopropyl-1-thio- $\beta$ -D-galactopyranoside (1.0 µM final concentration) was added to the ice-cold cultures, and then the cells were cultivated at 16 °C for 16 h. All following procedures were performed at 4 °C. The cells were collected by centrifugation at 5,000 × *g* and resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 300 mM NaCl and 10% (v/v) glycerol (buffer B). The cells were disrupted by sonication, and the lysate was centrifuged at 20,400 × *g* for 20 min at 4 °C. The supernatant was loaded onto a *Strep*-Tactin<sup>®</sup> Superflow agarose column (Novagen) equilibrated with wash buffer (buffer B containing 1 mM EDTA). After washing the resin with wash buffer, CalL-*strep* was eluted with buffer B containing 2.5 mM desthiobiotin. The buffer of the eluate, containing contaminating *E. coli* BL21(DE3) chaperones, was exchanged to 250 mM NaCl in 50 mM phosphate buffer (pH 7.0) using an Amicon 10,000 MWCO centrifugal filter. CalL-*strep* was finally purified by chromatography on a PROTEIN LW-803 column, under the same conditions used for the last step of the native phosphatase purification. The chromatogram is shown in Fig. S5B. The eluate was filtered using an Amicon 10,000 MWCO centrifugal filter to remove the inorganic phosphate and to concentrate the protein in buffer A.

#### Functional analysis of recombinant CalL and its mutants

The reaction mixture (50  $\mu$ L), containing 50 mM Tris-HCl, pH 8.0, 6  $\mu$ M phosphocalyculin A and 10 nM CalL-*strep* or its mutants, was incubated at 25 °C for 3 min, and then quenched by the addition of 50  $\mu$ L MeOH and centrifuged at 20,300 × *g* for 10 min. A 20  $\mu$ L aliquot of the supernatant was analyzed by diode array-detected (DAD) RP UPLC (Cosmosil 2.5C<sub>18</sub>-MS-II; 2.0 mm I.D. × 100 mm, Nacalai Tesque), using a linear gradient elution of 30% (0 min) to 100% (5-10 min) CH<sub>3</sub>CN in H<sub>2</sub>O containing 0.05% trifluoroacetic acid, at a flow rate of 0.4 ml/min. Phosphocalyculin A (retention time: 3.2 min) and calyculin A (4.0 min) were detected by UV absorption at 343 nm. The relative activity (%) of phosphocalyculin phosphatase was defined as follows: [Area values of calyculin A] / ([Area values of calyculin A] + [Area values of phosphocalyculin A]) × 100 (%).

#### Biochemical characterization of recombinant CalL

To determine the pH optimum, phosphocalyculin phosphatase activity measurements were performed in various buffers (pH range 2–9), including glycine-HCl (3.65), acetate (4.5), MES-NaOH (6.0–7.0) and Tris-HCl (7.25–9.0). Each reaction mixture (50  $\mu$ L) contained 50 mM buffer, 6  $\mu$ M of phosphocalyculin A and 10 nM CalL-*strep*.

Various phosphatase inhibitors (imidazole, phosphate, EDTA, EGTA, sodium fluoride, sodium molybdate, and sodium tartrate) were added to the enzymatic reaction catalyzed by the recombinant CalL-*strep*. Each reaction was initiated after a pre-incubation with various concentrations (0, 0.5 or 1.0, 5.0 or 10, 20 mM) of inhibitors for 10 min on ice. The reaction conditions and phosphatase activities were calculated according to the same method described in the functional analysis section.

To determine the steady-state kinetics parameters of native and recombinant CalL, various concentrations of phosphocalyculin A (final Conc. 2.5, 5, 10, 20, 100, 2000, 20000  $\mu$ M) were added to the solution (50  $\mu$ L) of 10 nM recombinant or native CalL in 50 mM Tris-HCl, pH 7.25. Immediately after an incubation at 25 °C for 60 sec, the reaction was quenched by the addition of 50  $\mu$ L MeOH and analyzed by UPLC, under the same conditions as described in the functional study section. The enzymatic product, calyculin A, was detected by monitoring the UV absorption at 343 nm and the area value of the calyculin A peak (retention time 4.01 min) was measured. The concentration of calyculin A was calculated by reference to the standard curve for calyculin A. Reactions were performed in triplicate. The  $K_m$  and  $k_{cat}$  values were estimated by the Michaelis-Menten equation and the curve-fitting program in KaleidaGraph<sup>\*</sup>.

#### Determination of metal ion content in CalL

Qualitative metal ion analysis by inductively coupled plasma-mass spectrometry, ICP-MS (Agilent), was used to determine the metal ion contents of the native and recombinant CalL, as well as the series of variants with a mutation in their putative metal-binding site. Buffer B without enzyme was also analyzed as the background. The net emission intensities of the samples for the presence of various metals were determined. The counts per second of each sample with various metals (Mg, Ca, Mn, Fe, Co, Ni, Cu, Zn) were adjusted by subtracting the value of buffer B, and the abundance of representative metals is shown in Fig. S9B. The presence of Cu and Zn ions in the wild-type recombinant CalL and its mutants (D116A, Y168F, Y168A) was further quantified by using a Metallo assay kit (Metallogenics), according to the manufacturer's protocol. After the addition of a Cu or Zn chelating chromophore to the samples, the absorbances of copper (OD<sub>583</sub>) and zinc (OD<sub>560</sub>) were measured by a microplate reader, respectively. The concentration

of Cu or Zn was calculated by reference to the standard curve for each metal. The molar ratios of protein to metal concentrations are shown in Table S4.

### Subcellular localization of the recombinant CalL

To specifically extract the periplasmic proteins, the lysozyme extraction method<sup>[4]</sup> was employed. The cultured cells heterologously expressing CalL were prepared according to the protocols in the functional analysis sections. The cell pellet was suspended in cold lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 20% [v/v] sucrose), treated with 3 mg of lysozyme and lysed by an incubation at 37 °C for 30 min. The lysates were centrifuged at 15,000 × g for 15 min, and the supernatants were loaded onto *Strep*-Tactin<sup>\*</sup> for purification. The eluate was concentrated using an Amicon 10,000 MWCO centrifugal filter and analyzed by 10% SDS-PAGE (Fig. S10).

### Localizations of phosphocalyculin phosphatase and calyculin A in D. calyx

To investigate the localization of phosphocalyculin phosphatase and calyculin A in D. calyx, we prepared enriched cell fractions by density gradient centrifugal fractionation. A 1.0 g portion of the lyophilized D. calyx tissue stored at -30 °C was transferred into a 50-mL Falcon tube and homogenized with 10 mL of Ca- and Mg-free artificial sea water (CMF-ASW). After the supernatant was decanted into another Falcon tube, 10 mL of CMF-ASW was added to the residue, and the homogenization process was repeated. The collected supernatants were centrifuged at 560  $\times$  g for 10 min to precipitate the sponge and bacterial cells. The cell fraction was washed twice with 35 mL of CMF-ASW, and then resuspended in 2.5 mL of 80% (v/v) PercolI<sup>™</sup> PLUS (GE Healthcare) in CMF-ASW. The cell suspension was loaded on a density gradient of Percoll™ PLUS solution, composed of 2.5 mL each of Percoll™ PLUS solutions (60%, 40% and 20% [v/v] in CMF-ASW). By centrifugation at 560 × q for 10 min, the cells were separated according to the difference in cell density (Fig. S11A). Ten fractions (1.0 mL each) were obtained from the top to bottom of the solution, by using a P1000 pipet. Each fraction was observed by microscopy (magnification ×200-400, Fig. S11B). The filamentous Entotheonella cells were enriched in fraction 3, whereas the sponge cells were observed in fractions 6-10, and small bacterial cells were concentrated in fraction 1. A 100 µL portion of each cell fraction was extracted with 100 µL of MeOH. The metabolic profiles of the MeOH extracts were analyzed by UPLC (same conditions as described in the functional analysis section). To examine the phosphocalyculin A phosphatase activity, 6 µg of phosphocalyculin A was treated with 2 µL of the enzyme extract of each fraction at room temperature for 5 min. After quenching with 78  $\mu$ L of MeOH and subsequent centrifugation, the enzymatic products were monitored by UPLC and the phosphatase activities were calculated according to the same method as described in the functional analysis section (Fig. S11C).

### Visualization of free phosphoric acid concentrations in "Candidatus Entotheonella" sp. cells

To visualize the location of the dephosphorylation reaction inside the bacterial producer cell, two Entotheonella spp. belonging to the same genus were used in this study. One is the producer of CalL and phosphocalyculins, the Entotheonella cells from the marine sponge *D. calyx*. The other is the bacterial symbiont "*Candidatus* Entotheonella" sp. in the marine sponge *D. kiiensis* as a negative control. The 16S rRNA sequence amplified from the filamentous bacterial cell fraction enriched from *D. kiiensis* showed 96% identity to "*Candidatus* Entotheonella" sp. associated with *D. calyx* (Fig. S12). We confirmed that *D. kiiensis* does not show any wound-activated enzymatic reactions or metabolic changes in the extracts on the basis of LC-MS analysis. At first, after the rehydration of the lyophilized sponge tissue with CMF-ASW (activation of enzyme activity) to prevent unwanted cell aggregation, the Entotheonella cells were collected by centrifugation at 200 × g for 10 min. To visualize the concentration of free phosphoric acid inside the cells, the bacterial cells were suspended in 80  $\mu$ L of CMF-ASW and stained with 20  $\mu$ L of malachite green reagent for 2 h at room temperature, and then observed by bright field microscopy (×400 magnification) (Fig. 4).

### SEM sample preparation and analysis

To observe the surface of Entotheonella cells by scanning electron microscopy (SEM), fresh *D. calyx* tissue, collected in the ocean by Izu Peninsula, Japan, in November 2020, was fixed in Davidson's fluid (22.2% of a 10% formalin solution in CMF-ASW, 32% ethanol, 11.1% glacial acetic acid, and 34.7% CMF-ASW) and kept at 4 °C until use. Slices with a thickness of 3–4 mm were cut from the middle portion of the tissue with a scalpel blade. These slices were dehydrated in graded ethanol followed by 100% *t*-butyl alcohol at 35 °C. After the slices were frozen in *t*-butyl alcohol at 4 °C, they were lyophilized and then coated with a 98% Au-2% Pd alloy (ion spatter, E101, HITACHI). Imaging was performed by field

emission-scanning electron microscopy (FE-SEM, JSM-6301F, JEOL). SEM images were captured under the following conditions (acceleration voltage, 5 kV; working distance, 6 mm).

# **Supplementary figures**

**Native CalL** 





To evaluate the isoelectric point (pl) of native phosphatase, the electrophoresis images of calibration marker (gray spots) was merged with the gel images of native and recombinant CalL (light blown spots; black allows), respectively. For calibration marker of pl, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4.7–8.3), carbonic anhydrase (CA; 4.7–6.7) and creatine phosphokinase (CPK; 4.7–6.0) were indicated in the image. Molecular weight marker (MM) was loaded on the right side of the gel.



### ESI-MSMS $m/z = 662.784 (M+2H)^{2+} CalL ('30-SDDYVWIELGK-40')$

# В

Α

ESI-MSMS  $m/z = 866.4 (M+2H)^{2+} CalL ('48-VITQSEACPNITVDGK-63')$ 





D

С

ESI-MSMS  $m/z = 610.2 (M+2H)^{2+} CalL ('71-STTPPEGFDIR-81')$ 



9

# Ε

ESI-MSMS m/z = 563.0 (M+H)<sup>+</sup> CalL (\*82-VCER-85')

# F

ESI-MSMS *m/z* = 365.2 (M+2H)<sup>2+</sup> CalL ('86-ELPAATK-92')



# **G** ESI-MSMS $m/z = 383.7 (M+2H)^{2+} CalL ('99-KPLPLAK-105')$









ESI-MSMS  $m/z = 329.1 (M+2H)^{2+} CalL ('121-VFTYK-125')$ 



12



# Κ

262.02

0 \ 20







O ESI-MSMS  $m/z = 373.7 (M+2H)^{2+} CalL ('379-SPSITNK-386')$ 



Ρ

ESI-MSMS *m/z* = 787.4 (M+2H)<sup>2+</sup> CalL ('392-ILQTLGVAPADFISE-406')



# Q

ESI-MSMS  $m/z = 317.7 (M+2H)^{2+} CalL ('434-KKFVLK-439')$ 



# Fig. S2. Comparisons of MS/MS fragmentation patterns of digested peptides from native protein and recombinant CalL.

MS/MS fragmentation of peptide fragments of the native and recombinant CalL were shown in the order of N to C terminal amino acid sequence of CalL (**A**–**R**). These peptide fragments were detected by ESI-MS/MS in positive mode. Upper and lower panels show the spectra from native and recombinant CalL, respectively. Neutral loss corresponding to an amino acid in MS/MS data was illustrated as one letter notation of amino acid.



# Fig. S3. Re-sequencing of the upstream of ORF calL

The upstream region of *calL* was re-sequenced, and the results were aligned together with the deposited sequence (accession code: AB933566.1). The sequence alignment was generated using Genius Prime 2020 software. The fosmid<sup>[2]</sup> containing the upstream regions of *calL* gene (green bar indicates *calL* gene ORF) were re-sequenced using the specific primer (Upst-CalL\_F, Table S2) (n = 2). The red triangles indicated that the duplicated adenine error at 15,215 bp in the upstream of ORF *calL*. After the removal of this error, the ORF of *calL* is revised to be 15,058–16,410. The amino acid sequence of the revised ORF CalL was shown in Fig. 2C.

	1	10	20	30	40	50	60	70	80	90	100
Ca. Entotheoneila spCall.: Nitrospirae bacterium_KWT18177.1: Duganeila sacchari SHN24114.1: Janthinobacterium sp. WP [099418073.1: Massilia umborata WP_137314957.1: Burkholderia pyrrocinia_WP_04703050.1: Cyanotheces sp. ACL47440.1: Ascidiaceihabitans donghaensis_WP_108828071.1: Consensus:	HPYALI MIFKLI	MEALAHAS MKNRFLRS ML AAARAAAPPS MRLLPPRF MRRLLPRAL AAGSGLAVL1 r	GRSFHYLFLA SLLCHIFILP LLSTLKRGAI SLLPPALLAL TRPYFASLA LARCAPLVA IFALIMLPNR 11KLTLALTA	LSYLSACSAY- FLVQCGGRAVE ALLLTGCAATN SALLAGCAGLF AAILAGCA-TF LAALSACSNHJ SPIVAADPQPL FGLMASQASAG la.c	SASDD PPHAAPYI PSPPPD PPAAPAGAGD PLPPDV DTPADPASAA DRPAASS FKGNIY	YVHIEL YKHYEL FTSYVVL FASFVVL LSTHVVL INVQAAHVEJ YPFVIH AGHVQY	GKGGVRIVR GPNGSITAR GEYGAAVAR GPEGAAVAR GEEGAATAR GDANQAIAR GPDG-AIAR TGDGTAQAR g++g+a+aR	VIT AVT VLT VIT VITNYTPAS CAIPT VVINTDPAN VVINTDPAN	-QSEACPNIT -KGAYCPDAV -DAPACPAIV -AAPACPAIV -AAPACPAIE -VAAACPTLE ASDPLCPQLT QTPIPCPNLC PAGTKCPDIF CP	TOSKSLAMQA TIDSKPYPMRI TIDSKPYPMRI TIDSKPYPMRI TIDGRARPHTL DGRARPHTL VDGRPYPMAY VDGKLSRHTL LNNSPPQAMQ RHGNTKTPHTL .dpm.	RSTTP RYVKE RAPAQTI RAPAATL RAPAATL RAGAATR IHAAPTL RSGFAPL F
Ca, Entotheonella spCal.: Nitrospirae bacterium _KWI78177.1: Duganella sacchari _SHN24114.1: Janthinobacterium spWP_09310937.1: Massili aumhonata WP_137314957.1: Burkholderia pyrrocinia WP_047903503.1: Cyanothece spACL47440.1: Ascidiaceihabitans donghaensis _WP_10828017.1: Consensus:	101 I ALR APRRAI AQR AQR 	110 ATPSAPF OAKAAAKAKF STASSPF PTASDPF	120 KGEHEY JOSKPAEFPL JVAKAAAFPL LOSKPSAFFY DOSKPSNFPY NFPY 	130 RVCERELPAAT TVCETDIPAEF LTCETPLPAGT LSCEAALPAGT LACEAPLPAGT SVCETTLPADF QVCQLGIPKNV RVCVAPVDSTP .vCe.lpa.	140 KSVVFA-KKP KTVSIE-STP TSASVL-GRA ASASVL-GRA QKAALA-GIE QAASVA-GRT TSASIN-QQP VGMFVPPALR as	150 LPLAKRKPR LPLLKKSPS LPLLKKSPS LPLPRHEAR LPLPRHEAR LPLPRHEAR LPLPKANPK LPLPKANPK LPIPKANPK LPIPKANPK LPIPKP.r	160 IVIIGDTGC VLIIGDTGC IVVIGDTGC IVVIGDTGC IIVIGDTGC IALIADTGC IALVGDTGC IAVLGDTGC i.!igDTGC	170 RVFTYKTHY RISDD RLQKSSN RLKRSDDGD RLKRADH RIKKADN RIEGN RVAYG R	180 DVQNCNGVEC ELQACNN SHQACND SYQACND AHQACND AHQACND LQQSCNN IYQNCTG-DC QaCn	190 YGPAHPFPKI SSAHVFPDI SQAYPFATT REQYPFATY PEQFPFAGY PEQFPFAGY PQAPFAQL GGKAHDYEAY 	200 
Ca, Entotheonella spCal.: Nitrospirae bacterium_KWI78177.1: Duganella sacchari_SHN24114.1: Janthinobacterium spWP_0931093.1: Massilia umbonata WP_131314957.1: Burkholderia pyrrocinia_WP_047903503.1: Cyanothee spACL47440.1: Ascidiaceihabitans donghaensis_WP_108828017.1:	201 RPDLI KPDLI KPDLY RPELY RPDLY SPDLY KPDLY APDLF PDLY	210 CHLGDYHYRE CHNGDYTYRC CHVGDYHYRE CHVGDYHYRE CHVGDYHYRE CHVGDYHYRE CHVGDHHYRE CHVGDHHYRE	220 TPCPKGNKG SLCPYESDG NACPDGNAG HPCPAGNAG NPCPDGNAG NACPPDIAG GQC-SKNPD TGTCDEG	230 CRGSPSGFTHF CRGSPHGYGDNHC CRGSPHGYGHC CRGSPHGYGHC CRGSPHGYGHC CRGSPYGOGHC CRGSPYGOGHC CRGSPYGOGHC CRGSPYGOGHC CRGSPSHG2AHC	240 SHEADFFAPA AHDADFFAPG AHDADFFAPG AHNADFFTPG THREDFFRPA THQADLFRPA SHKADFFDPS LH, ADFf.Pa	250 RDLL TRAPHY QNLL PAAPHJ ARLL DAAPHJ ARLL DAAPHJ ARLL RAAPHY EPLL RAAPHY APLL AKAPHY APLY ARAPHY ATYF QTAPLJ 	260 FIRGNHESCI FLRGDRENCI MARGNHENCI VARGNHESCI VYRGNHEECI VIRGNHEDC RG#bE.C	270 ARAHLGHFY ALNPYGHFR LRGGQGYHR ARAGQGHFR ARAGQGHFR TRAHKGHFY Arag, GHFr	280 LLOPNPLPA- LLOPFPSE- FLOPRPLLKC FLOPRPLQAC FLOPRPLQAC FLOPRPYSAF LLOPRPKPA- LLOPRPFEA1 LLOPRPIEA1	290 R R R R	300 
Consensus.											
Ca, Entotheonella sp. Cal.: Nitrospirae bacterium KWI781771: Duganella sacchari_SHN2114.1: Janthinobacterium y.WP_099418073.1: Massilia umbonata WP_137314957.1: Burkholderia pyrrocinia WP_047903503.1: Cyanothece sp. ACL47440.1: Ascidiaceihabitans donghaensis_WP_108280171.1: Consensus:	301 I SQCPA CEN DDVGN DAVGD DATGD DATGD DTNAN -QCPD GNDHS	310 SEPYNVLL SEPYNVLL SEPYNVLL SEPYAVPLC SEPYAVPLC SEPYAVPLC TOPYAVPAC TOPYAVPAC TLPYPVTFC SEPYA!,1	320 SLQLLVQDS JTLTLSVLDS GATQLLVLD GODQLLVLD GGDQUIVYDD G-PLNLVVLD DSFQVIVHDT glvv.d	330 SGYHYSPKO TSANDDTYSKY TANTTHKGLKF TAATSHRGLKF TAATSHRGLKF TAATSHRGLKF TAATSHRGLKF TAASHRGLKF AATSHRGLKF SSLAA-DSKF ARIDNDYGSRF	340 HEASVALYTK KSSRYASYLK GDVGYARYQD DSLGYRRYQQ GDTGFDRYRD TDAQFGIYQK PPAQVQQYRT DQGAVDQYKY Y.	350 VYNEVNKVS TYENTD LYRKHAALSO AYRQLDALAR AYRQLDALAR QFQTVATLAS QFQTVATLAS QFQTTAKLTK EFSATELTAE ,%1.	360 -SKHRNF QAPNNLA RASHNIG RTTHNLLI KAGMTTTIF KNTHLI QAA-YPSHL'	370 LTHEPIMAI LTHRPIMAY IAHHPLLGH WHHPLLGF ANHHPVLGF INHHPILAF LTHRPIMAF VTHRPFMAI LTHRPFMAI	380 QPGATPQGTY TEOPNIENKI GADRKADGSJ GAERGADGAY GAGLDKASQY APIAGSTPA- ATATN ASYGSEAAP-	390 LYPLQLT EDKLFTLNHT VLLPGDAG PGNLA PGNLA PGNLA PGNLA PGNLA PGNLA	400 LQAALKK LQTAFRS LQAAMGS LQAFGA LQQSFGS LQSYMSS LQKAVKD LQVALAQ LQ.a
Ca. Entotheonella sp. Cal.: Nitrospirae bacterium_KWT18177.1: Duganella saccharf SHN21114.1: Janthinobacterium sp. WP 099418073.1: Massilia umborata WP 107903003.1: Carnothece sp. ACL47440.1: Ascidiaceihabitans donghaensis_WP 108828017.1: Consensus: Ca. Entotheonella sp. Cal.: Nitrospirae bacterium_KWT78177.1: Duganella saccharf_SHN2114.1: Janthinobacterium p. WP 099418073.1: Burkholderia pyrocinia WP 107930503.1: Casentotheonella sp. Cal.: Nassilia umborata WP 107930503.1: Casentotheonella sp. Cal.: Burkholderia pyrocinia WP 047903503.1: Cyanothece sp. ACL47440.1: Ascidiaceihabitans donghaensis_WP 10882801741: Consensus: Ca. Entotheonella sp. Cal.: Casensus: Ca. Entotheonella sp. Cal.:	301 1	310 ISOPYPISLE SEPYNVUL SEPYNVUL SEPYNVUL SEPYNVUL SEPYNVUL SEPYNVUL SOPYNVL	320 SLOLL VODS TIL TLSVLDS SOTTILL VLD SOTTILL VLD SOTTILL VLD SOTTILL VLD SOTTILL VLD SOSTILV VLD S	330 SGUNYSPKC TSANDDYSK TRATSHRGLK TRATSHRGLK TRATSHRGLK TRATSHRGLK A30 CHETD-G-RPF FSTRF5C-HP VSFSRUS VSFSR-US VSFSSRUS VSFSSR-US VSF	340 HERSVALYTK KSSRYRSYLK GOVGYRSYLD GOVGYRSYLD GOVGYRSYLD GOVGYRSYLD GOVGYRSYL 440 HHVZGNSGTK GYZGNSGTK G	350 VYNEVNKRYS TYENTD	360 -SSENIS -SKIRUF -SKIRUF -STWAL -SENITG	370 LTHEPIAG THRPIAG CHIPLLGM CH	380 <b>QPGATPQGTU</b> <b>TEDPNIENK</b> <b>GRDRAGOS</b> <b>GRDRAGOS</b> <b>GRDRAGOS</b> <b>GRDRAGOS</b> <b>GRDRAGOS</b> <b>HIGTU</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b> <b>480</b>	390 	400 

# Fig. S4. Sequence alignment of CalL and its homologs

Accession codes of NCBI were indicated after species names except for CalL. High (< 90%) and low (< 50%) consensus regions were highlighted as red and blue, respectively. The sequence alignment was carried out using free web tool, MultAlin.<sup>[6]</sup>



### Fig. S5. Purification of recombinant CalL using LW-803

- (A) 10% SDS-PAGE of recombinant CalL-strep (black triangle) expressed in *E. coli* BL21(DE3), roughly purified by Strep-Tactin<sup>®</sup> affinity chromatography. Lane M; molecular weight ladder, Lane 1; insoluble proteins, lane 2; soluble proteins, 3; flow-through, 4; eluted fraction.
- (B) Gel-filtration profile of the crude sample from Strep-Tactin<sup>®</sup> purification (lane 4 in left figure). Sample was fractionated by PROTEIN LW-803 column. Column elutes were monitored by UV absorption at 280 nm. The four fractions were obtained (retention time of Fr. 1; 4.0–6.5 min, Fr.2; 6.5–10.0 min, Fr. 3; 10.0–12.0 min, Fr. 4; 12.0–17.0 min, Lane B; before purification) and analyzed on 10% SDS-PAGE.



# Fig. S6. Biochemical characterization of CalL

- (A) Partial HPLC chromatograms of enzymatic reaction catalyzed by recombinant CalL-*strep* with phosphocalyculin A. As a negative control, boiled CalL-strep was used (bottom).
- (B) CalL relative activity measured at various pH values. Glycine-HCl (pH 3.65), acetate (pH 4.5), MES-NaOH (pH 6.0–7.0) and Tris-HCl (pH 7.25–9.0) were used as buffers.
- (C) Effects of phosphatase inhibitors on recombinant CalL. All data were collected in triplicate (*n* = 3).



Fig. S7. Michaelis-Menten plots of native and recombinant CalL reaction with phosphocalyculin A.

CalL steady-state kinetic analysis with phosphocalyculin A. Data are presented as mean values +/- SD.





# Fig. 8. Phylogenetic relationships between MPE domains

- (A) The cladogram was built with the seed alignment of the MPE superfamily from Pfam (PF00149; containing 324 sequences in addition of CalL and 7 homologs) using the neighbor-joining algorithm in MEGA-X.<sup>[7,8]</sup> Branches were grouping and colored on the basis of their functions of the representative enzymes in the clade, respectively. The newly generated CalL clade was indicated as red color. The colored dots indicate the homologs listed in Fig. 3 were marked with spots.
- (B) Enlarged view of PAPs and CalL branches. Pfam codes and the regions of MPE domain were indicated. In the case of CalL clade, accession codes of NCBI and the regions of MPE domain were described.



Fig. S9. Identification of metal-coordinating sites and catalytic metal ions of CalL

- (A) The relative phosphocalyculin A phosphatase activities of the CalL mutants. All data were collected in triplicate (n = 3).
- (B) Qualitative analysis of metal ions for native, recombinant CalL and its mutants by ICP-MS.



### Fig. S10. Localization of recombinant CalL in E. coli

10% SDS-PAGE of the CalL-*strep* fractions purified on the *Strep*-Tactin<sup>\*</sup> column (Lane: Elute) from periplasm (*left*) and whole cell (*right*). The recombinant CalL-*strep* is indicated with red triangles, whereas the co-purified chaperones (DnaK and GroES) are indicated with black triangles. Lanes S and FT represent soluble and flow-through fractions, respectively.



- (B) Representative images of cell fractions under a brightfield microscope, filamentous Entotheonella cells were pointed out with orange arrows.
- (C) Phosphatase activity against phosphocalyculin A and content of calyculin A for each fraction, -; substrate phosphocalyculin A, +; crude enzyme solution of sponge (n = 3, means ± SEM).



### В

### >Entotheonella 16S rRNA partial D. kiiensis (as query)

BLAST results	Host marine sponges	Identity	Accession
Candidatus Entotheonella palauensis	Theonella swinhoei	97.15%	AF130847.1
Candidatus Entotheonella sp.	Discodermia calyx	96.57%	AB683979.1
Candidatus Entotheonella sp.	Discodermia dissoluta	97.04%	AY897125.1
Candidatus Entotheonella factor	Theonella swinhoei	96.95%	KF926817.1

### Fig. S12. Detection of Candidatus Entotheonella sp. in Discodermia kiiensis

- (A) Acquisition of 16S rRNA sequence from the filamentous cell fraction of *D. kiiensis*. First, the 16S rRNA regions were amplified from DNA of the filamentous cell fraction by PCR with universal 16S rRNA primers (Table S2). Subsequently, three combinations of *Entotheonella*-targeting primers were used against the PCR products to amplify 16S rRNA sequences of Entotheonella spp. (Table S2). The PCR products of 271F–1309R was sequenced. Lane M: DNA marker.
- (B) The 16S rRNA sequence of the filamentous cell fraction and its homology search results by using the nucleotide basic local alignment search tool (BLASTn).



- Fig. S13. SEM micrographs of Entotheonella cells in *D. calyx*.
- (A) SEM micrograph of *Ca*. Entotheonella sp. in sponge tissue. Yellow allows indicate some of the Entotheonella cells (scale bar,  $10 \mu m$ ).
- (B) A disrupted Entotheonella filament (scale bar,  $1 \mu m$ ).
- (C and D) Enlarged views of the area surrounded by the frame in B (scale bar, 100 nm, PM; portions of peeling off the membrane).
- (E) Another example of a wounded Entotheonella cell.
- (F) Intact Entotheonella cells (scale bars of E and F; 1  $\mu m).$

# Supplementary tables

ORF	<i>pl/</i> MW (kDa)	Proposed function	Sequence similarity	Identity/ Similarity (%)	Accession code
CalY	5.35/87	AT	Malonyl CoA-acyl carrier protein transacylase	54/71	ZP_11129640.1
CalX	4.37/9	ACP	Acyl carrier protein	57/79	YP_001613822.1
CalW	5.70/43	KS <sup>0</sup>	Beta-ketoacyl synthase	65/81	ZP_15538843.1
CalV	4.55/14	Hypothetical protein	Hypothetical protein ACP_1149	39/63	YP_002754252.1
CalU	6.03/79	ABC transporter	ABC-type multidrug transporter	44/66	YP_007079509.1
CalT	4.80/47	HMG-CoA synthase	3-hydroxy-3 methylglutaryl CoA synthase	70/80	ZP_18905080.1
CalS	5.76/30	Enoyl-CoA hydratase	Enoyl-CoA hydratase/isomerase	61/73	ZP_08192880.1
CalR	7.17/27	Enoyl-CoA hydratase	Polyketide biosynthesis enoyl-CoA hydratase	63/77	YP_003871374.1
CalQ	5.26/43	Putative phosphotransferase	Aminoglycoside phosphotransferase	30/41	ZP_06974247.1
CalP	5.64/26	Putative phosphotransferase	Chloramphenicol 3-O phosphotransferase	31/52	ACS83703.1
CalO	5.12/30	Glyoxalase	Glyoxalase/bleomycin resistance protein/dioxygenase	38/51	ZP_13490391.1
CalN	5.92/48	Hypothetical protein	Hypothetical protein BTH_II1215	28/47	YP_439411.1
CalM	4.72/26	Putative phosphotransferase	Chloramphenicol phosphotransferase	31/47	ZP_09089298.1
CalL	9.01/43	Protein phosphatase	Phosphoprotein phosphatase	40/52	YP_002485801.1
CalK	5.84/44	Transposase	Transposase IS4 family protein	48/64	YP_004693916.1
CalJ	6.78/63	Hypothetical protein	Unknown protein	78/85	AFS60661.1

 Table S1. Physicochemical properties of putative proteins in calyculin BGC.

Table S2. List of	primer sequences.
-------------------	-------------------

Primer	Orientation	5'-Sequence-3'
Upst-CalL_F	Forward	TTCACATGCCGGGTGC
NL_CalL_Eco_F2	Forward	c c <u>gg aat tcA</u> ATG GTG GTA GTC ACT <u>CAT ATG</u> G
CalL-St-HillI_Re	Reverse	ccc <u>aag ctt</u> cta <i>ctt ttc gaa ctg cgg gtg gct cca gct tgc</i> TTT TAG CTG GCT TTT AGA AAC
CalL_D116A_F	Forward	TT GTC ATC ATC GGC <b>GCT</b> ACC GGT TGC CGC G
CalL_D116A_R	Reverse	C GCG GCA ACC GGT <b>AGC</b> GCC GAT GAT GAC AA
CalL_D165A_F	Forward	C ATT CAC CTT GGC <b>GCT</b> TAT CAC TAC CGC GA
CalL_D165A_R	Reverse	TC GCG GTA GTG ATA <b>AGC</b> GCC AAG GTG AAT G
CalL_Y168F_F	Forward	T GGC GAT TAT CAC <b>TTC</b> CGC GAG ACG CCC TG
CalL_Y168F_R	Reverse	CA GGG CGT CTC GCG <b>GAA</b> GTG ATA ATC GCC A
CalL_Y168A_F	Forward	T GGC GAT TAT CAC <b>GCC</b> CGC GAG ACG CCC TG
CalL_Y168A_R	Reverse	CA GGG CGT CTC GCG <b>GGC</b> GTG ATA ATC GCC A
CalL_N215A_F	Forward	C TTC ATC CGC GGC <b>GCT</b> CAC GAG AGT TGC GC
CalL_N215A_R	Reverse	GC GCA ACT CTC GTG <b>AGC</b> GCC GCG GAT GAA G
CalL_H305A_F	Forward	C TGG TCG CTG ACC <b>GCT</b> GAA CCC ATC TGG GG
CalL_H305A_R	Reverse	CC CCA GAT GGG TTC <b>AGC</b> GGT CAG CGA CCA G
CalL_H352A_F	Forward	TT CTT CTG AGC GGG <b>GCT</b> GTG CAT CTG TTC G
CalL_H352A_R	Reverse	C GAA CAG ATG CAC <b>AGC</b> CCC GCT CAG AAG AA
CalL_H354A_F	Forward	TG AGC GGG CAT GTG <b>GCT</b> CTG TTC GAG TCC T
CalL_H354A_R	Reverse	A GGA CTC GAA CAG <b>AGC</b> CAC ATG CCC GCT CA
16S_27F <sup>9</sup>	Forward	AGAGTTTGATCMTGGCTCAG
16S_1492R <sup>9</sup>	Reverse	GGTTACCTTGTTACGACTT
Ento271F <sup>10</sup>	Forward	GGGAAASGTTCGCBGGTCTG
Ento735F <sup>10</sup>	Forward	GYATTAAGCCKYGGAAACKGT
Ento1290R <sup>10</sup>	Reverse	GCCCRGCWYVACCCGGTA
Ento1309R <sup>10</sup>	Reverse	GCGTKCTGATCTCCGATTAC

The restriction sites (*Eco*RI, *Nde*I and *Hind*III) were underlined, *Strep* tag II (for CalL-St-HiIII\_Re primer) was described as italic. The mutation sites were indicated as bold letters.

Enzyme	K <sub>m</sub> (μM)	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	<i>k</i> <sub>cat</sub> / K <sub>m</sub> (M <sup>-1</sup> ⋅ s <sup>-1</sup> )
Native phosphatase	27.4 ± 2.94	4.2 ± 0.10	$(1.53 \pm 0.15) \times 10^5$
Recombinant CalL	26.7 ± 8.93	5.1 ± 0.66	(1.91 ± 0.17) × 10 <sup>5</sup>
Recombinant CalQ	501 ± 35.0	0.134 ± 0.01	(2.76 ± 0.55) × 10 <sup>2</sup>

Table S3. Steady-state kinetics parameters of enzyme activation/inactivation.

Phosphocalyculin A and calyculin A were used as substrates for the phosphatase CalL and the phosphotransferase CalQ, respectively. All reactions were performed in triplicate (n = 3).

	Protein	Cu	Zn
wild-type CalL	1.00	0.74	0.88
D116A	1.00	0.15	0.09
Y168F	1.00	0.14	N.D.
Y168A	1.00	0.05	0.11

### Table S4. Molar ratios of wild-type CalL or its mutant to metal ion.

The concentrations of Cu and Zn ions were determined by using Metallo assay kits. (N.D. = not detected)

### References

- [1] A. Takai and G. Mieskes, *Biochem. J.*, 1991, **275**, 233–239.
- [2] T. Wakimoto, Y. Egami, Y. Nakashima, Y. Wakimoto, T. Mori, T. Awakawa, T. Ito, H. Kenmoku, Y. Asakawa, J. Piel and I. Abe, Nat. Chem. Biol., 2014, 10, 648– 655.
- [3] A. Shevchenko, H. Tomas, J. Havlis, J. V. Olsen and M. Mann. Nat. Protoc., 2006, 1, 2856–2860.
- [4] K. Oikawa, H. Tomas, J. Havli, V. J. Olsen and M. Mann, J. Biol. Chem., 2005, 280, 5527–5532.
- [5] R. Keren, B. Mayzel, A. Lavy, I. Polishchuk, D. Levy, S. C. Fakra, B. Pokroy and M. Ilan, Nat. Commun., 2017, 8, 14393.
- [6] F. Corpet, Nucleic Acids Res., 1988, 16, 10881–10890.
- [7] R. D. Finn, A. Bateman, J. Clements, P. Coggill, R. Y. Eberhardt, S. R. Eddy, A. Heger, K. Hetherington, L. Holm, J. Mistry, E. L. L. Sonnhammer, J. Tate and M. Punta, Nucleic Acids Res., 2014, 42, D222–D230.
- [8] S. Kumar, G. Stecher, M. Li, C. Knyaz and K. Tamura, *Mol. Biol. Evol.*, 2018, **35**, 1547–1549.
- [9] G. W. Weisburg, S. M. Barns, D. A. Pelletier and D. J. Lane, J. Bacteriol., 1991, 173, 697–703.
- [10] M. C. Wilson, T. Mori, C. Rückert, A. R. Uria, M. J. Helf, K. Takada, C. Gernert, U. A. Steffens, N. Heycke, S. Schmitt, C. Rinke, E. J. Helfrich, A. O. Brachmann, C. Gurgui, T. Wakimoto, M. Kracht, M. Crüsemann, U. Hentschel, I. Abe, S. Matsunaga, J. Kalinowski, H. Takeyama and J. Piel, Nature, 2014, 506, 58–62.