Supporting Information

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SI Materials and Methods

Cultivation and Immune Stimulation of Amphioxus and Preparation of Tissue Samples. Adults of Chinese amphioxus (*Branchiostoma japonicum*) were obtained from Qingdao, China then maintained in large tanks with aeration and supplied with fresh seawater daily. Tissues were separated under an optical microscope (Nikon). For immune stimulation, live bacteria in PBS suspension (1×10^8 cfu/mL) were injected into the coelom of amphioxus (15 μL per animal). PBS was injected as the negative control. Hepatic ceca and intestine from five animals per treatment were collected at 2, 4, 8, 12, 24, and 48 h after injection and were frozen as experimental samples using liquid nitrogen. Total RNA was extracted using TRIzol (Invitrogen), and the sample was subjected to DNase I treatment (Promega) according to the manufacturer's protocols. Subsequently, reverse transcription was performed using the PrimeScript RT reagent kit (Takara). For oral infection, amphioxus was maintained in filtered seawater and *Staphylococcus aureus* or *Escherichia coli* was added to the seawater to a final concentration of $\sim 1 \times 10^7$ cfu/mL.

Isolation of the Full-Length cDNA of *Branchiostoma japonicum* Apextrin-Like Proteins. Expressed sequence tags with sequence similarity to Apextrin were identified from a suppression subtractive hybridization library constructed by our laboratory (1). To obtain complete cDNA sequences, 3′ and 5′ RACE was performed using the GeneRacer Kit (Invitrogen) with gene-specific primers according to the manufacturer's instructions. The amplified fragments were cloned into the pGEX-Teasy vector (Promega) and were sequenced using an ABI PRISM 3730 DNA analyzer. Two full-length cDNA sequences consisting of 504 and 462 aa residues were obtained and were designated *Branchiostoma japonicum* apextrin-like proteins 1 and 2 (bjALP1 and bjALP2), respectively.

Quantitative Real-Time PCR Analysis of the Gene Expression Patterns for bjALPs. Quantitative real-time PCR was performed on a LightCycler 480 real-time PCR system (Roche) using the SYBR Premix Ex Taq II kit (Takara) in a 10-μL reaction system. A total of 45 PCR cycles were run, and the melting curve was recorded. The data were quantified using the $2^{-\Delta\Delta Ct}$ method based on the Ct values of *bjALPs* and *bjβ-actin*. All of the samples were analyzed in three replicates, and the results are expressed as the mean ± SD.

Section in Situ Hybridization. A 514-bp fragment of bjALP1 and a 532-bp fragment of bjALP2 were cloned into pGEM-Teasy (Promega). Digoxigenin-labeled sense and antisense probes for bjALP1 and bjALP2 were synthesized using a DIG RNA Labeling Kit (Roche). The animals were dehydrated with gradated ethanol, embedded in paraffin, and sliced into 8-μm transverse histological sections. After drying at 42 °C overnight the sections were dewaxed, rehydrated, washed, and digested by proteinase K. The sense or antisense probes were added to the sections and hybridized overnight at 42 °C. After high-stringency washing the sections were immunodetected using an NBT/5-bromo-4-chloro-3- indolyl phosphate stock solution (Roche).

Sequence Retrieval, Alignment, and Domain Prediction. The protein sequences of the *Branchiostoma floridae* and *Heliocidaris erythrogramma* apextrins were used to perform BLASTP searches against all of the available databases at the National Center for Biotechnology Information (NCBI) and the Joint Genome Institute (JGI, http://genome.jgi-psf.org/Brafl1/Brafl1.home.html). Domain structure predictions were performed on the main page of the SMART website (http://smart.embl-heidelberg.de). For sequence analysis, the ALP protein sequences were first aligned using ClustalX 1.83 (2) and were manually corrected using GeneDoc (3). A minimum-evolution (ME) tree was built by using Mega v4.1, with 1,000 bootstrap tests and handling gaps by pairwise deletion.

Preparation of Recombinant Proteins. PCR fragments encoding amino acids 20–504, 20–297, and 298–504 of bjALP1 and 21–462, 21–257, and 258–462 of bjALP2 were inserted into plasmid pET-32a (+) (Novagen). The recombinant expression plasmids were introduced into *E. coli* BL21 (DE3) for protein expression. All six fusion proteins in inclusion bodies were denatured in 8 M urea and were renatured in three dialysis steps as previously described (4). Each dialysis step was performed at 4 °C for at least 12 h followed by desalting with Tris-buffered saline (TBS) buffer (50 mM Tris·Cl and 150 mM NaCl, pH 8.0) using a Sephadex G-25 column. The concentration of the purified protein was determined using a Bio-Rad Protein Assay dye reagent and BSA as a standard.

Microbial Binding Assay. A total of 5 μg of purified recombinant proteins were incubated with microbes in 1 mL of TBS buffer at 4 °C overnight with end-over-end mixing. The mixtures were centrifuged at 12,000 × *g* for 1 min at 4 °C, and the pellets were washed five times with 1 mL of TBS buffer. To denature the binding proteins, the washed pellets were suspended in 100 μL of TBS buffer [50 mM Tris·Cl (pH 7.5) and 150 mM NaCl] and 20 μL of 6× loading buffer and boiled at 100 °C for 10 min. Western blotting was performed with an anti-His mouse monoclonal antibody (Sigma) to validate the binding proteins.

Microbial Aggregation Assay. Fluorescein isothiocyanate (FITC) (isomer I; Amersco) was used to label bacteria. Suspensions of bacteria were mixed with 50 μL of FITC solution (10 mg/mL in DMSO) and gently agitated at room temperature in the dark for 3 h. After washing five times with TBS, FITC-labeled bacteria were mixed with 10 μg ALP proteins in 1 mL of TBS and were incubated at room temperature in the dark for 2 h. Bacterial aggregation was examined immediately using fluorescence microscopy (Carl Zeiss).

Analysis of ALPs Binding with Microbial Cell-Wall Components. ELISA was used to analyze the the binding of ALP proteins with soluble microbial cell wall components as previously described (5). In brief, LPS, lipoteichoic acid, peptidoglycans (PGNs), mannan, zymosan (Sigma), and muramyl dipeptide (MDP) (Invivogen) in carbonate buffer (0.1 M NaHCO3 and 2.5 mM Na2CO3, pH 9.6) were used to coat 96-well microtiter plates (TPP) for 3 h at 37 °C (20 μg each well, PGN and zymosan are ultrasonically solubilized). After washing with TBS three times, the wells were blocked with TBST (0.05% Tween 20 in TBS) containing 10% (wt/vol) BSA overnight at 4 °C. Various concentrations of ALP proteins were then added, and the mixtures were incubated for 2 h at 37 °C. Binding proteins were detected using a mouse anti-His mAb followed by incubation with HRP-labeled anti-mouse IgG (Bio-Rad). The absorbance was read at 450 nm immediately after being developed with the 3,3,5,5′-tetramethyl-benzidine substrate (Sigma). The assay was repeated three times. The insoluble PGN-binding activities of ALP proteins were detected by pull-down assays as previously described (5). In brief, insoluble PGNs were incubated at 4 °C

for 1 h with 5 μg of ALP proteins in TBS. The samples were centrifuged at 15,000 × *g* for 10 min and pellets were washed with the TBS, centrifuged, and suspended in 100 μL of TBS buffer and 20 μL of 6× loading buffer and boiled at 100 °C for 10 min. Western blotting was performed with an anti-His mouse monoclonal antibody (Sigma) to validate the binding proteins.

Antimicrobial Activity Assays. Thirty milliliters of 1.0% warm nutrient agar mixed with *S. aureus* were poured into a 90-mm plate. The pores are 0.5 cm in diameter perforated with perforex. Then, the targeted protein or antibiotic in 20 μL of TBS was added to the pores at a final concentration of 1 μg/μL. The plates were incubated at 37 °C for 16 h. A transparent ring around the pores indicated antibacterial activity. The growth curves of *S. aureus* were tested as follows. Two single colonies were picked up and transferred into 1 mL of Luria–Bertani (LB) broth. A volume of 50 μL of cell suspension was mixed with 200 μg targeted proteins and added to 1 mL LB broth. Each sample was incubated at 200 rpm at 37 °C and OD_{600} was measured every 1 h.

Preparation of Antibodies. Anti-bjALP1 and Anti-bjALP2 mouse monoclonal antibodies were prepared against the epitopes ^{412}GEVPDGNYDR^{421} and ^{375}DGIYNRNTEI^{384}, respectively, by Abmart Company using the SEAL technology.

Expression Plasmids. For the study of subcellular localization, full-length bjALP2 was inserted into the pEGFP-N1 vector (Clontech). For the reporter assays and coimmunoprecipitation (Co-IP) test, full-length bjALP2 was fused with a Flag tag on the C terminus and cloned into pcDNA3.1 (+) (Clontech). PCR fragments encoding amino acids 1–257 and 258–462 of bjALP2 were fused with a Flag tag on the C terminus, cloned into pcDNA3.1 (+), and designated as bjALP2ΔC and bjALP2ΔN, respectively. The bjMyD88 sequences were cloned into pCMV-HA (Clontech), bjTRAF6 (TNF receptor-associated factor 6) sequences were cloned into pCMV-Myc and pCMV-Flag (Clontech), and bjUbiquitin was cloned into pCMV-HA (Clontech).

Immunofluorescence Imaging. HeLa cells seeded onto coverslips (10 × 10 mm) in a 24-well plate were transfected with 400 ng of the indicated expression plasmids. Twenty-four hours later, the cells were fixed for 15 min in a 4% (wt/vol) formaldehyde solution (in PBS), washed three times in PBST (0.05% Tween-20 in PBS), and blocked with 5% (wt/vol) BSA in PBST at room temperature for 1 h. The cells were then incubated with 1 μg/mL of a mAb for 1 h, washed three times in PBST, and incubated with the secondary antibody at 4 °C overnight. After triple washing in PBS, cells were labeled with 0.2 μg/mL DAPI in PBS for 5 min and were washed three times in PBS. The cells were then mounted in MOWIOL R4-88 Reagent (Calbiochem) and photographed with a Carl Zeiss Axiovision 4 microscope.

Immunogold Assay. Hepatic ceca were fixed for EM in a mixture of glutaraldehyde and paraformaldehyde in seawater overnight and embedded in Epon resin. Sections (60 nm) were collected on 200-mesh nickel grids. Samples were incubated overnight at 4 °C with anti-ALP1 or anti-ALP2 antibody. Washes were followed by 1 h of incubation at room temperature in goat anti-mouse secondary antibody labeled with 20-nm gold particles. For excrement immungold assay, 1×10^9 *S. aureus* in 100 mL filtered seawater were used to orally infect amphioxus for 4 h; the excrement was collected and prepared for EM as described above.

H&E Staining. Animals were fixed with 4% (wt/vol) paraformaldehyde in PBS overnight at 4 °C. Samples were embedded in paraffin following dehydration in ethanol. Tissues were cut into 8-μm sections and stored for staining. For H&E (Sigma) staining, sections were freed of paraffin and stained.

Survival Analysis. Twenty amphioxi were maintained in 100 mL filtered seawater and *S. aureus* and *Vibrio anguillarum* was added to the seawater to a final concentration of ~1×10^7 cfu/mL with or without 0.5 μg/mL bjALP1 antibody. Dead amphioxi were counted at indicated time points after bacterial oral infection. Purified IgGs from preimmunized mouse serum were used as control.

Transfection and Reporter Assays. HEK293T cells were plated in 48-well plates (at 5×10^4 cells per well) and transfected 24 h later with a mixture of DNA as previously descripted (6). The mixed DNA contained the indicated amounts of expression vector, 5 ng of pRL-TK Renilla luciferase reporter plasmid (Promega), 50 ng of the pNFκB-Luc (Clontech), and the complementary empty vector. Luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were normalized to *Renilla* luciferase activities and are expressed as the fold stimulation relative to that measured in cells transfected with empty vector. To determine the effect of MDP on NF-κB activities, variant amounts of MDP were co-transfected with indicated plasmids to HEK293T cells. Each experiment was performed at least in triplicate and was repeated at least twice in all cases.

Co-IP. HEK293T cells in six-well dishes were transfected with 6 μg DNA plasmids (3 μg/each expression vector). At 36 h post-transfection, whole-cell extracts were prepared in IP lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid sodium salt, and a protease inhibitor mixture from Roche] and incubated with primary antibodies (1 μg anti-Flag; Sigma) at 4 °C for 4 h then incubated with Protein G Sepharose (Roche) at 4 °C overnight. The next day, the mixture was washed three times with lysis buffer. The analysis was conducted using SDS/PAGE followed by Western blotting with anti-Myc (1:7,000) and anti-Flag (1:1,000) mouse monoclonal antibody (Sigma). To assess the impact of MDP on the interaction of bjALP2 and TRAF6, an indicated amount of MDP was added to the cell lysate of HEK293T cells that transfected with bjALP2 and TRAF6 plasmids.

Statistical Analysis. Quantitative data are presented as the mean ± SD and compared statistically by two-tailed Student *t* test. A *P* value of < 0.05 was considered statistically significant.

1. Huang G, et al. (2007) Profile of acute immune response in Chinese amphioxus upon Staphylococcus aureus and Vibrio parahaemolyticus infection. *Dev Comp Immunol* 31(10):1013–1023.
2. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. *Annu Rev Immunol* 21:335–376.
3. Gerold G, Zychlinsky A, de Diego JL (2007) What is the role of Toll-like receptors in bacterial infections? *Semin Immunol* 19(1):41–47.
4. Yu XQ, Tracy ME, Ling E, Scholz FR, Trenczek T (2005) A novel C-type immulectin-3 from Manduca sexta is translocated from hemolymph into the cytoplasm of hemocytes. *Insect Biochem Mol Biol* 35(4):285–295.
5. Yu Y, et al. (2007) A short-form C-type lectin from amphioxus acts as a direct microbial killing protein via interaction with peptidoglycan and glucan. *J Immunol* 179(12): 8425–8434.
6. Yuan S, et al. (2009) An amphioxus TLR with dynamic embryonic expression pattern responses to pathogens and activates NF-kappaB pathway via MyD88. *Mol Immunol* 46(11–12):2348–2356.

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A
  1 MAVWGCTAVS LLLVALCQGA PTEVVQKETS DDIFKFVPEA LELYDTGKGN
 51 IYDYMDIILA PEVGLQTLPN STKIPDGSLS REKRVAILAT LAAVAPIASA
101 VFGAGSFIYG IINGQQRLAE LREINRKLDR LDRKLDQLQQ QLGDVQFGQQ
151 WLEGAVLYGR DIQRLNYFIN YLDNNLNLGS NGRLVPANQA NNWANAVLHL
201 GSDGVGQVLL NLNDMMMDTS GIFGRNSLFV LYESRLDQDS DDYWPKVRQF
251 LEFAFSIQTA GYASWATALN IKGRQGEVAA VVARGRRGLD AQRQFLQRFT
301 KQWPTGTYGL PRTNTGCPVA AGARWRTGVR KHDTEDDDAS NQWTNGLHFD
351 GEFGRNNMKQ KFCMKTSSGD GDGNWPRGSY CIFKKGGCPR GFQSGEIYWD
401 DEDDDNGNSR SGEVPDGNYD RNTLIKYCCR NDGSANNQIS LPNRSPFYLF
451 RYRQGCQKVA GMNVREEFFR WDDEDDDNKN RRRGAHPYDD GGNSNHRLHY
501 CYYS*
B
  1 MKFALLVGVF VLSIICQTQS APTELQAAEF DYGQEAAHPR KRRSILAVVI
 51 PIAVNLLQQG IEAYGRQKAQ QLEQDQLEAL RDIQRKLDVL DRKVDALQRA
101 IGELRFGQQW LEGAVLYGND IQRLEYFLDF LDRRLSPGNN GQLVPNNLAE
151 EWADAVLSLD DDGAGQVLHN LHEMIIGSSG LFGRNSLFVL YESTLDGQST
201 QYWPKVRQFL DFTFSIQVAG YAAWVTSLNI KDRSLQEVAE VIDTANTRIE
251 EQRVFLLPYT KEWPRGSYGL PRTNTGCPVA ANARWLNGVR HHDTEDDDNS
301 NAWIGGIHFD GGYGTNMDQK FCMKTDTAMG EGNWPAGNYC IFKKGNCPVG
351 FQWGKLKWDD EDDDNKNSAS GVLPDGIYNR NTEIQYCCRS DGSATTPIAL
401 PSRRPFYLFR FRAGCQRVAD MAVREEWFRW DDEDDDNANR YYGAHPFDQG
451 GRNHRIHYCY YS*
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Fig. S1. Amino acid sequences of bjALP1 (*A*) and bjALP2 (*B*). The putative signal peptide is in italics, the boxed sequence indicates the predicted TRAF6 binding motif, and the underlined sequence indicates the APT-C region. The asterisk indicates the stop codon. The GenBank accession number of the corresponding nucleotide sequence is KM017614.

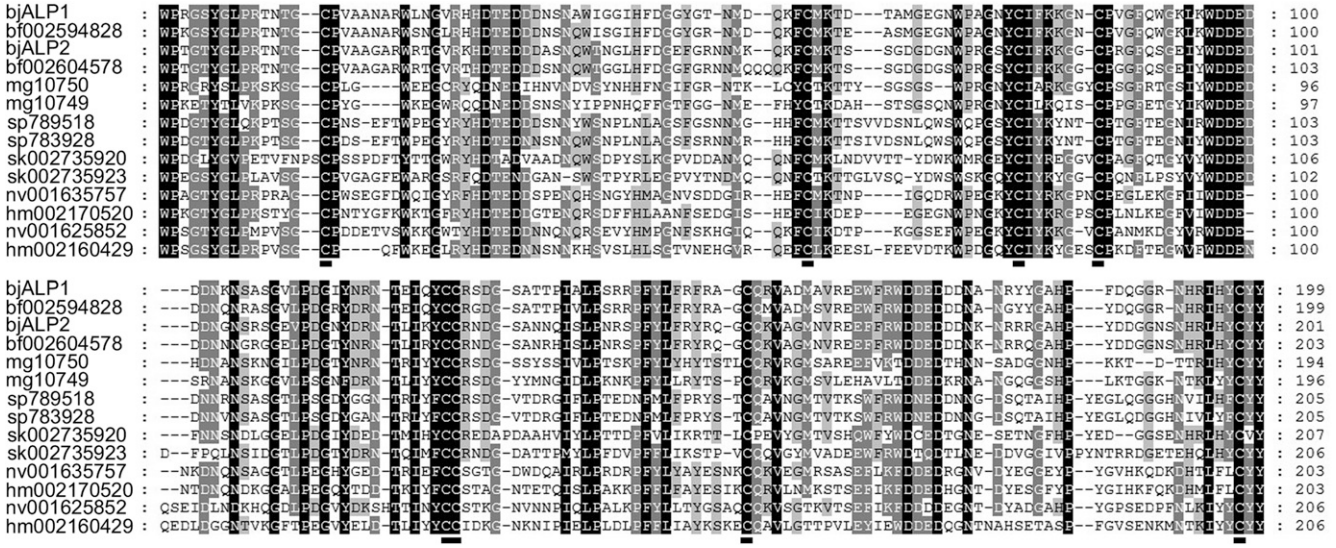

Fig. S2. Sequence alignment of the apextrin C-terminal (ApeC) domain. The eight conserved cysteine residues in character are underlined. am, *Acropora millepora*; bf, *Branchiostoma floridae*; he, *Heliocidaris erythrogramma*; hm, *Hydra magnipapillata*; is, *Ixodes scapularis*; mg, *Mytilus galloprovincialis*; nv, *Nematostella vectensis*; pl, *Pomatoceros lamarckii*; sk, *Saccoglossus kowalevskii*; sp, *Strongylocentrotus purpuratus*. The sequence used in the alignment and phylogenetic analysis is indicated as follows: bf002594828 (XP_002594828); bf002604578 (XP_002604578); mg10750 (AEK10750); hm00217052 (XP_00217052); hm002158419 (XP_002158419); hm002165606 (XP_002165606); nv001635757 (XP_001635757); nv001627111 (XP_001627111); am091848 (EF091848); he28440 (AAC28440); sp001179344 (XP_001179344); sp789518 (XP_789518); sp786957 (XP_786957); sk002735920 (XP_002735920); sk002735923 (XP_002735923); pl11402 (ADB11402); and is002400395 (XP_002400395).

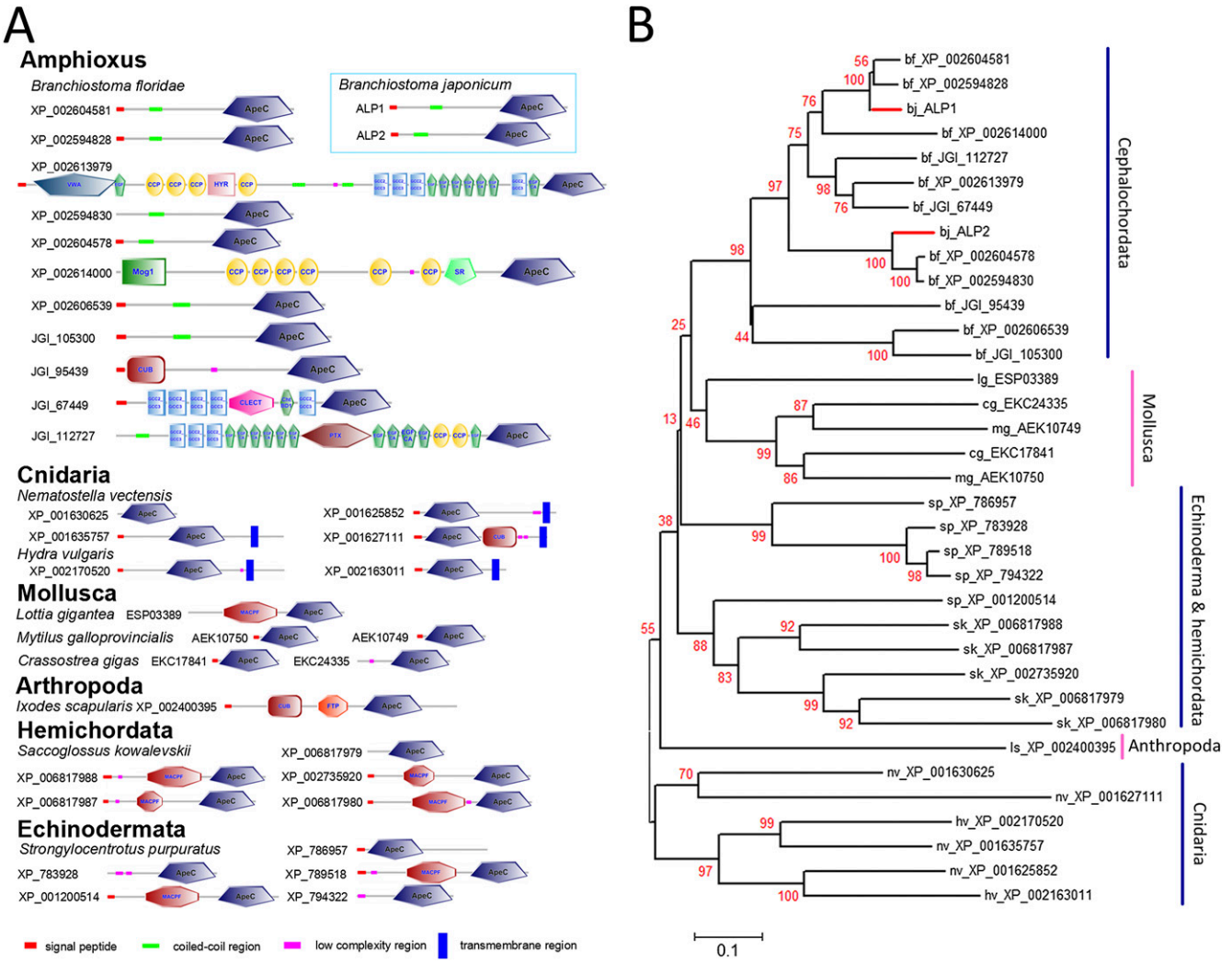

Fig. S3. Domain architecture and phylogeny of apextrin-like proteins. (*A*) ApeC domain-containing ALP proteins in amphioxus and ApeC domain-containing ALPs selected from other animal phyla. (*B*) The ME tree of the ApeC domain protein sequences of various ALPs from different phyla or subphyla.

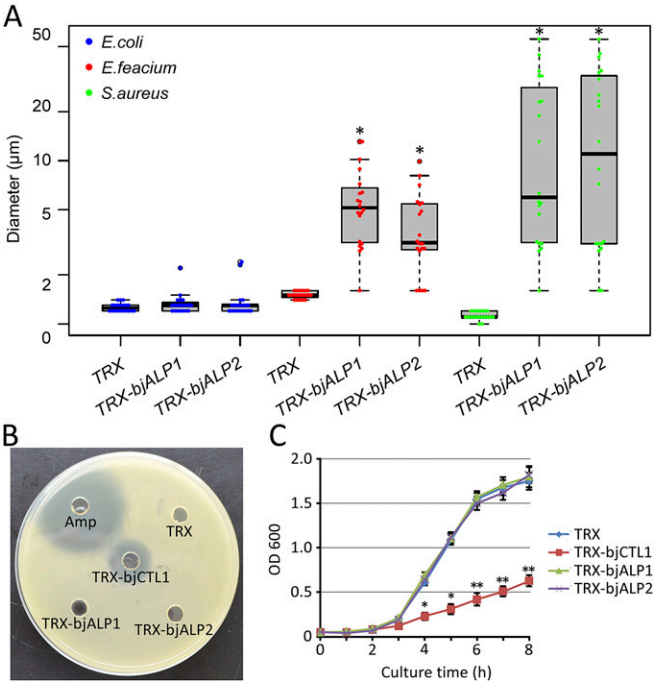

Fig. S4. Antibacterial activity test of ALP proteins against *S. aureus*. (*A*) Box plot showing the diameters of green puncta in bacteria aggregation assays. Box plot explanation: upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile. Circles represent outliers. *$P < 0.05$ versus TRX control. (*B*) A photograph of a plate of a representative agar plate assay with 1 μg/μL ampicillin (Amp), 0.2 μg/μL TRX-bjCTL1, 1 μg/μL TRX, 1 μg/μL TRX-bjALP1, or 1 μg/μL TRX-bjALP2. Individual recombinant proteins or ampicillin were applied to pores within the transparent agar plates with microbes. Then, the plates were incubated at 37 °C for 16 h. The conversion of the otherwise transparent agar into an opaque yellowish appearance provided an indicator of the active growth of the bacteria. (*C*) The results of the growth curve assay. *$P < 0.05$.

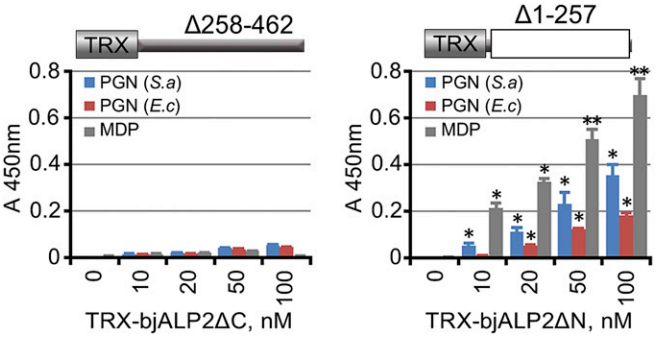

Fig. S5. ELISA analysis of the interaction between the truncated bjApextrin1 mutants and PGN and MDP. One representative experiment of six is shown. Background absorbance without protein was subtracted.

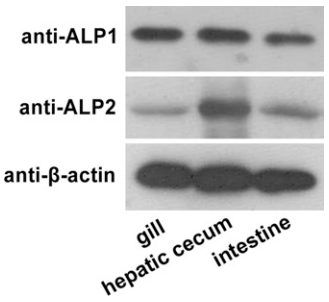

Fig. S6. Western blot analysis of bjALPs in different tissues. Gill, hepatic cecum, and intestine were collected from 10 amphioxi and suspended in 100 μL of PBS buffer and 20 μL of 6× SDS/PAGE loading buffer and the tissues were lysed by boiling at 100 °C for 10 min. Western blotting was performed with anti-ALP1 or anti-ALP2 mouse monoclonal antibodies.

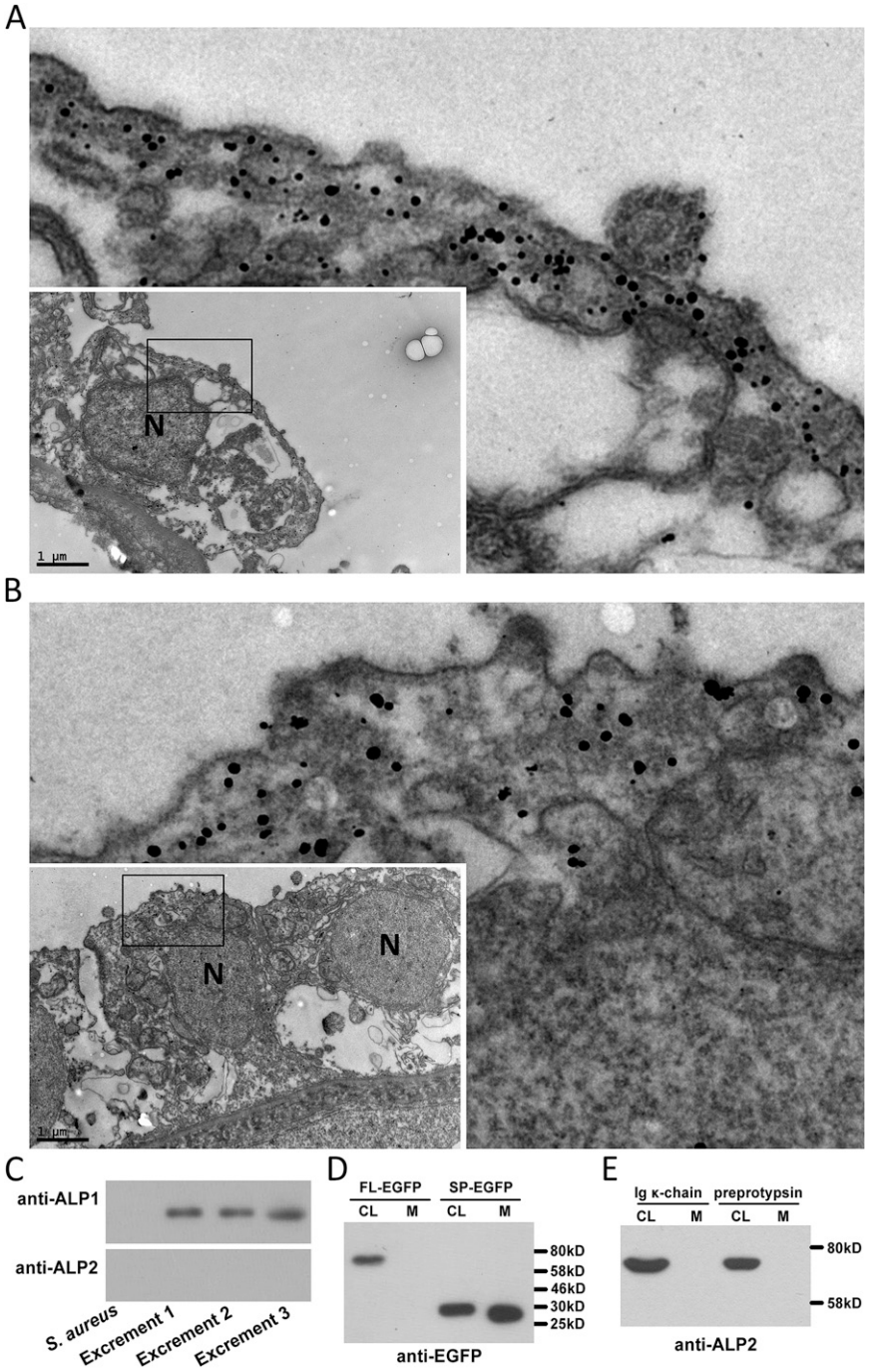

Fig. S7. Immunogold EM analysis and Western blot of biALPs. (*A* and *B*) Immunogold EM analysis of bjALP2 in the hepatic cecum (*A*) and intestine (*B*) of amphioxus. N, nucleus. (*C*) Western blot of bjALPs in bacteria excrement. 1×10^9 *S. aureus* in 100 mL filtered seawater were used to orally infect amphioxus for 4 h. The excrement was collected and suspended in 100 μL of PBS buffer and 20 μL of 6× SDS/PAGE loading buffer and then boiled at 100 °C for 10 min. Western blotting was performed with anti-ALP1 or anti-ALP2 mouse monoclonal antibodies. (*D*) Western blot analysis of bjALP2-GFP fusion protein overexpressed in HEK293T cell. Anti-GFP antibody was used. FL, full-length ALP2; SP, signal peptide of ALP2. (*E*) Western blot analysis of mature bjALP2 peptides fused with different signal peptides overexpressed in HEK293T cells. Anti-bjALP2 antibody was used. CL, cell lysates; M, culture media.

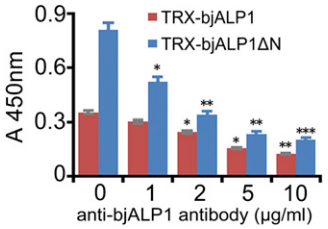

Fig. S8. ELISA analysis showed that anti-bjALP1 antibody can block the interaction between recombinant ALP proteins and MDP. Background absorbance without protein was subtracted.

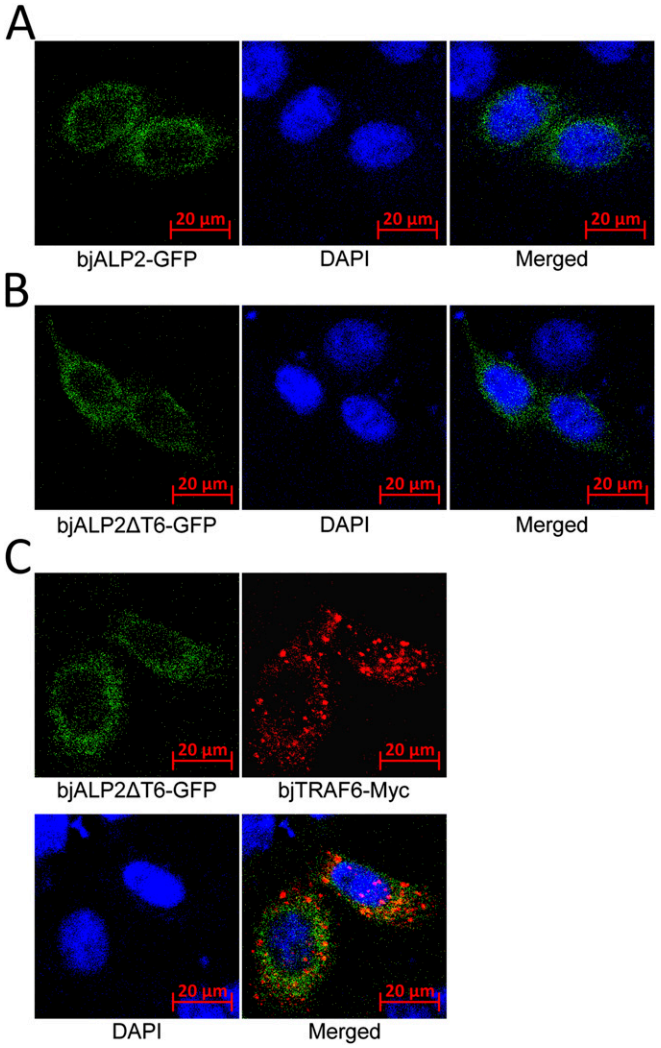

Fig. S9. Subcellular location of bjALP2 and its mutant. (*A*) Subcellular location of EGFP-fused bjALP2. (*B*) Subcellular location of EGFP-fused bjALP2ΔT6. (*C*) BjALP2ΔT6 is not colocalized with amphioxus TRAF6 in HeLa cells. HeLa cells were cotransfected with EGFP-fused bjALP2ΔT6 and Myc-tagged pCMV-bjTRAF6 and were stained with an anti-Myc antibody and an Alexa Fluor 532 secondary antibody.

Table S1. Primer list

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size, bp
	Quantitative real-time PCR primers		
bjALP1	CATGGCGGTTTGGGGGTGTA	ACAGCTCTAGCGCCTCGGGAAC	131
bjALP2	TGCCAAACCCAGTCCGCTCC	GGGATGACCACTGCGAGGAT	107
β-actin	TCTGGCATCATACCTTCTACAA	TCTGTGTCATCTTTTCCCTGTT	113
	RACE primers 5′–3′		
bjALP1 3′RACE	TTCCGTTGGGACGATGAAGATG		
bjALP1 5′RACE	CGTCATCTTCATCGTCCCAACGGAA		
bjALP2 3′RACE	TTCCGGTGGGACGATGAAGAC		
bjALP2 5′RACE	CGTCTTCATCGTCCCACCGGAA		
	Cloning primers for in situ hybridization		
bjALP1	gcggtttgggggtgtact	ccaggttcaggttgttgtcc	532
bjALP2	ggaacgtcagaacattcacca	cctgctccatcgtcgtcc	514
	Cloning primers for pET-32a (+)		
bjALP1	CGCGGATCCgcaccgaccgaagtcgtaca	CCGCTCGAGagagtagtagcagtagtg	1,473
bjALP1ΔC		CCGCTCGAGctgtttggtaaaccgttggagg	852
bjALP1ΔN	CGCGGATCCcaaggtgaagttgcggctgt		639
bjALP2	CGCGGATCCgctccgaccgagctgcaag	CCGCTCGAGcgaatagtaacagtagtgga	1,326
bjALP2 ΔC		CCGCTCGAGttctttagtgtagggcagaagg	729
bjALP2 ΔN	CGCGGATCCagcctgcaggaggtggcag		633
	Cloning primers for pcDNA3.1 (+) and/or pEGFP-N1		
bjALP2	CGCGGATCCatgaaattcgcgctgctc	CCGCTCGAGcgaatagtaacagtagtgga	1,386
bjALP2 ΔC		CCGCTCGAGttctttagtgtagggcagaagg	789
bjALP2 ΔN	CGCGGATCCagcctgcaggaggtggcag		633