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Involvement of the cell-specific pigment genes *pks* and *sult* in bacterial defense response of sea urchins *Strongylocentrotus intermedius*

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ABSTRACT: Bacterial infections are one of the most important problems in mass aquaculture, causing the loss of millions of juvenile organisms. We isolated 22 bacterial strains from the cavity fluid of the sea urchin *Strongylocentrotus pallidus* and used phylogenetic analysis based on 16S rRNA gene sequences to separate the bacterial strains into 9 genera (*Aliivibrio*, *Bizionia*, *Colwellia*, *Olleya*, *Paenibacillus*, *Photobacterium*, *Pseudoalteromonas*, *Shewanella*, and *Vibrio*). Incubating *Strongylocentrotus intermedius* larvae with a strain from each of the 9 bacterial genera, we investigated the viability of the larvae, the amount of pigment cells, and the level of polyketide synthase (*pks*) and sulfotransferase (*sult*) gene expression. Results of the assay on sea urchin development showed that all bacterial strains, except *Pseudoalteromonas* and *Bizionia*, suppressed sea urchin development (resulting in retardation of the embryos' development with cellular disorders) and reduced cell viability. We found that *pks* expression in the sea urchin larvae after incubation with the bacteria of 9 tested genera was significantly increased, while the *sult* expression was increased only after the treatment with *Pseudoalteromonas* and *Shewanella*. Shikimic acid, which is known to activate the biosynthesis of naphthoquinone pigments, increased the tolerance of the sea urchin embryos to the bacteria. In conclusion, we show that the cell-specific pigment genes *pks* and *sult* are involved in the bacterial defense response of sea urchins.

KEY WORDS: Echinochrome · Marine bacteria · Sea urchin · *pks* gene · *sult* gene · Pigment cells

INTRODUCTION

A variety of pathogens and parasites from protozoans to chordates (Jangoux 1990) can infect echinoderms, but bacterial disease is one of the most critical problems in commercial aquaculture. The sea urchin *Strongylocentrotus intermedius* has become the dominant and most economically important maricultured species in North China (Wang et al. 2012); however, sea urchins from a variety of geographical areas suffer from the spectacular sea urchin balding disease (Scheibling & Stephenson 1984, Jangoux 1990). This disease progresses from a discoloration of the epidermis that surrounds the spines, to the loss of spines and other appendages followed by the loss of epidermis and superficial dermal tissues, and finally to destruction of the skeleton and the appearance of lesions on the aboral surface (Scheibling & Stephenson 1984).

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This communicable disease is caused by different marine bacteria species, e.g. *Vibrio* sp., *Flexibacter* sp., or *Aeromonas* sp. (Gilles & Pearse 1986, Tajima et al. 1997a,b, Becker et al. 2007, 2008) and can result in mass mortalities that affect 10 to 90% of sea urchin populations in nature and in aquaculture. Once infected, sea urchins do not seem to recover, and there is no known cure. Additionally, a disease of *S. intermedius* characterized by lethargy in activity, a blackish, peristomial membrane, and body-wall lesions has frequently occurred in recent years. Affected animals also exhibit a high mortality rate (Wang et al. 2012).

Infections of sea urchins in small-scale aquaculture may be avoided by treating individuals for 1 to 2 h with antibiotics such as gentamycin (10 mg l^{-1}), neomycin (30 mg l^{-1}), novobicin (30 mg l^{-1}), or sulfisoxalole (0.25 mg l^{-1}) (Böttger et al. 2004). Unfortunately, no treatment has been discovered for bacterial infections in mass aquaculture, where millions of juvenile organisms (i.e. seed stock) have been lost to diseases (Böttger et al. 2004). In the present report, we investigated a native defense of the sea urchin *Strongylocentrotus intermedius* against marine bacteria and, specifically, the involvement of sea urchin polyketide compounds.

Polyketide compounds are a large group of structurally diverse, multifunctional proteins that are mainly found in bacteria, fungi, and plants. One of these polyketide compounds, the pigment echinochrome, is synthesized in the pigment cells of sea urchin larvae and adults (Griffiths 1965). These compounds and many marine secondary metabolites possess highly effective antioxidant, antibacterial, antifungal, antitumor, and psychotropic activities (Mishchenko et al. 2003, Cooper 2004), and these compounds may play a role in immune defense (Service & Wardlaw 1984).

Gene regulatory networks of sea urchin development have been characterized in detail by Davidson (2006). Previously, it has been found that the polyketide synthase (*pks*) gene and a sulfotransferase gene (*sult*) are specifically expressed in pigment cells, which suggests that these genes are required for biosynthesis of the pigment echinochrome (Calestani et al. 2003). Sea urchin embryos of *Strongylocentrotus purpuratus* lacking *pks* (via knock-down) develop pigment cells but appear unpigmented; these individuals are referred to as the albino phenotype (Calestani et al. 2003). Moreover, the addition of shikimic acid (ShA), which is a precursor of naphthoquinone pigments, to zygotes and embryos of sea urchins increased expression of the *pks* and *sult* genes (Ageenko et al. 2011).

This study focuses on a detailed gene expression profile for 2 cell-specific pigment genes of *Strongylocentrotus intermedius*, *pks* and *sult*, in embryos of the sea urchin *S. intermedius* cultivated with several strains of marine bacteria that were found in the cavity fluid of adult sea urchins from the Sea of Okhotsk. The possible involvement of the *pks* and *sult* genes in the defense of sea urchins against marine bacteria is discussed.

MATERIALS AND METHODS

Animals

Adult sea urchins *Strongylocentrotus intermedius* were collected in the Sea of Japan (Amursky Bay or Vostok Bay) and kept in tubes filled with running, aerated seawater. The animals were rinsed free of any debris with UV-sterilized, filtered seawater and injected with 2 to 3 ml of 0.5 M KCl to chemically induce spawning. The embryonic material was obtained by artificial fertilization and then placed in tanks with UV-sterilized seawater (17°C) throughout development until the mesenchymal blastula, gastrula, prism, or pluteus stages: 14, 24, 34, and 72 h post-fertilization (pf), respectively. The embryos were examined with an inverted microscope (Axiovert 200M, Carl Zeiss), with 10× and 20× dry objectives.

Bacterial strains

Bacterial strains used in the present work were obtained from the cavity fluid of adult sea urchins *Strongylocentrotus pallidus* (diameter of the testa: 6 to 8 cm) from the Sea of Okhotsk, near the Kuril Islands (Iturup, Simushir, Chirpoy; depth: 400 to 500 m) during the 41st scientific expedition of the research vessel 'Akademik Oparin' (July 2011). For strain isolation, 0.1 ml of the cavity fluid was transferred to agar plates with Difco Marine Broth 2216 (Becton Dickinson). After primary isolation and purification, strains were cultivated at 28°C on the same medium and stored at –80°C in marine broth supplemented with 20% (v/v) glycerol.

The phylogenetic affiliations of the bacteria were based on 16S rRNA gene sequences. Total DNA isolation and the PCR analysis were performed as described previously (Dubrovina et al. 2010, Kiselev et al. 2012). The primers p27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and p1524r (5′-AAG GAG GTG ATC CAR CCG CA-3′) were used to amplify the 16S

rRNA genes (Lane 1991). Amplification reactions were performed in volumes of 25 µl containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.1 mM Triton X-100, 0.2 mM of each dNTP, 0.2 µM of primer, and 1 U of *Taq* DNA polymerase (Silex M). Approximately 10 to 50 ng of DNA was used as a template. The analysis was performed in an UNO Thermoblock thermal cycler (Biometra) programmed for an initial denaturation step of 2 min at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s at 55°C (T_a), 2 min at 72°C, and a final cycle of 72°C for 5 min, using the fastest available transitions between each temperature.

PCR products were purified with ethanol and directly sequenced in the Instrumental Centre of Institute of Biology and Soil Sciences (Far Eastern Branch of Russian Academy of Sciences, FEB RAS) using an ABI 3130 Genetic Analyzer (Applied Biosystems) as described previously (Kiselev & Dubrovina 2010, Shumakova et al. 2011).

Susceptibility to antibiotics was tested by the conventional diffusion plate technique using Solid Medium A (0.2% [w/v] Bacto peptone [BD], 0.2% [w/v] Bacto yeast extract [BD], 0.1% [w/v] glucose, 0.002% [w/v] KH_2PO_4, 0.005% [w/v] $MgSO_4 \cdot 7H_2O$, 1.5% [w/v] Bacto agar [BD], 50% [v/v] natural seawater, and 50% [v/v] distilled water; pH 7.5 to 7.8), and discs were impregnated with the following antibiotics (per milliliter): ampicillin (10 µg), benzylpenicillin (10 U), carbenicillin (100 µg), cephalexin (30 µg), cephazolin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), ofloxacim (5 µg), oleandomycin (15 µg), oxacillin (10 µg), polymyxin (300 U), rifampicin (5 µg), streptomycin (30 µg), tetracycline (30 µg), and vancomycin (30 µg).

The following physiological and biochemical properties were examined: colony color, morphology, and sodium requirement (0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12% [w/v]) NaCl. The growth of bacteria at different temperatures was determined in Marine Broth 2216 after 24 to 72 h at 5, 10, 28, and 37°C. Some physiological and morphological characteristics of strains used are listed in Table 1.

Experiments with marine bacteria

Overnight solutions of bacterial cultures (+28°C, 10 000 000 CFU ml^{-1}) in Difco Marine Broth 2216 (Becton Dickinson) at the desired concentrations: 1 million (up to 100 000 CFU ml^{-1}) and 5 million bacteria cells (500 000 CFU ml^{-1}) per Petri dish (diameter 5 cm, with 10 ml of sterile seawater) were added to sea urchin zygotes (after 20 min pf: 200 zygotes ml^{-1}). Embryos and larvae were cultivated with the bacteria for 2 to 3 d. The development of the culture was monitored to ensure that the embryos were developing normally. Sea urchin zygotes without bacteria were used as controls. After this period, total RNA was isolated from the larvae for quantitative real-time PCR. Three independent experiments were performed to determine the effect of bacteria on sea urchin development.

RNA analyses

Total RNA from larvae of the sea urchin *Strongylocentrotus intermedius* at various stages of development was extracted with Yellow Solve reagent (Clonogen) and treated with DNase as described previously (Ageenko et al. 2011). Complementary DNAs were synthesized using 1 to 3 µg of RNA by the reverse transcription PCR Kit (Silex M). Reactions were performed in 50 µl aliquots of the reaction mixture, which contained reverse transcriptase (RT) buffer, 0.2 mM each of the 4 dNTPs, 0.2 µM of oligo-(dT)15 primer, and 200 U of M-MLV-polymerase, at 37°C for 1 to 2 h (Dubrovina et al. 2010, Tyunin et al. 2012). The 0.2 to 5 µl samples of reverse transcription products were then amplified by PCR of the sea urchin actin gene using the primers 5′-CAA CGG ATC CGG TAT GGT GAA GGC-3′ and 5′-TCC AGA CGG AGG ATG GCG TGG GGA-3′. The RT-PCR reactions were performed according to protocol (reverse transcription PCR Kit, Silex M) using an iCycler thermocycler (Bio-Rad Laboratories) with the following conditions: 1 cycle of 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 50°C, and 35 s at 72°C, with a final extension cycle of 15 min at 72°C. In the following real-time PCR analyses, we used only those reverse transcription reactions that resulted in 500 bp RT-PCR products for the actin gene. We discarded those reverse transcription reactions that resulted in both 500 and 700 bp RT-PCR products for the actin gene, which indicated DNA contamination.

Quantitative real-time PCR

Quantitative real-time PCR was used to measure the relative amount of *pks* and *sult* transcripts during the course of development and in specific tissues of the adults. Quantitative real-time PCR was performed

Table 1. Characteristics of bacteria used in experiments isolated from cavity fluid of the sea urchin *Strongylocentrotus pallidus* from the Sea of Okhotsk. +: positive; –: negative; ND: not detected

Characteristics	*Alii-vibrio* sp. TH-K1	*Bizionia* sp. TH-K2	*Colwellia* sp. TH-K3	*Olleya* sp. TH-K4	*Paeni-bacillus* sp. TH-K5	*Photobac-terium* sp. TH-K6	*Pseudo-altero-monas* sp. TH-K7	*Vibrio* sp. TH-K8	*Shewa-nella* sp. TH-K9
Colony color	Yellow	Yellow	White	Yellow	White	Cream	White	White	Yellow
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
NaCl range for growth (%, w/v)	2–4	2–6	2–4	1–4	0–6	1–4	0–8	1–8	0–4
Temperature (°C) range for growth	5–37	5–37	10–37	10–37	5–37	5–28	10–28	5–37	5–30
Most close species based on 16S rRNA gene sequences	100% to *Alii-vibrio logei* isolate AV04/2007, (EU257748)	100% to *Bizi-onia* sp. KMM 6177, (FJ716799)	99% to *Colwellia aestu-arii* strain SMK-10 (NR_043509)	99% to *Olleya marilimosa* strain CAM030 (EF660466)	99% to *Paeni-bacillus illino-isensis* strain JCM 9907 (NR_040884)	100% to *Photo-bacterium* sp. P65 (EU864266)	100% to *Pseudo-alteromonas elyakovii* (AB000389)	99% to *Vibrio kana-loae* strain HNS028 (JN128262)	99% to *Shewa-nella living-stonensis* clone SE86 (AY771775)
Antibiotic (conc.) susceptibility									
Ampicillin (10 µg ml^{-1})	+	+	–	+	+	–	+	+	+
Benzylpenicillin (10 U ml^{-1})	–	+	+	+	+	ND	+	–	+
Carbenicillin (100 µg ml^{-1})	+	+	+	+	+	+	+	+	+
Cephalexin (30 µg ml^{-1})	+	–	+	–	–	ND	+	+	+
Cephazolin (30 µg ml^{-1})	–	+	+	+	+	ND	+	+	–
Chloramphenicol (30 µg ml^{-1})	–	–	+	–	–	–	+	+	+
Erythromycin (15 µg ml^{-1})	+	+	+	+	+	–	+	+	+
Gentamicin (10 µg ml^{-1})	–	–	–	–	–	–	–	–	–
Kanamycin (30 µg ml^{-1})	+	–	–	–	+	+	–	–	–
Lincomycin (15 µg ml^{-1})	+	–	–	+	+	ND	+	–	+
Nalidixic acid (30 µg ml^{-1})	–	+	–	+	+	ND	–	+	+
Neomycin (30 µg ml^{-1})	–	+	–	+	+	+	–	–	+
Ofloxacim (5 µg ml^{-1})	–	–	+	+	+	ND	+	–	–
Oleandomycin (15 µg ml^{-1})	–	–	–	+	+	–	+	–	–
Oxacillin (10 µg ml^{-1})	–	–	+	–	+	ND	+	+	+
Polymyxin (300 U ml^{-1})	+	–	+	–	–	ND	+	+	–
Rifampicin (5 µg ml^{-1})	–	+	+	+	+	–	–	–	+
Streptomycin (30 µg ml^{-1})	–	–	+	–	+	ND	+	+	+
Tetracycline (30 µg ml^{-1})	–	+	–	+	+	–	+	–	+
Vancomycin (30 µg ml^{-1})	+	+	–	+	+	ND	+	–	–

using the established protocol (Ageenko et al. 2011). For TaqMan real-time RT-PCR, cDNAs were amplified in 20 µl of the reaction mixture containing 1× TaqMan Buffer B, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide, 1 U *Taq* DNA polymerase, 0.5 µl cDNA samples, and 0.25 µM of each primer and probe (Real-Time PCR Kit, Syntol). The amplification conditions consisted of 1 cycle of 2 min at 95°C, followed by 50 cycles of 10 s at 95°C and 25 s at 62°C. The TaqMan PCR assays were performed in an iCycler thermocycler supplied with the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories), and data were analyzed with the iQ5 Optical System Software V.2.0 according to the manufacturer's instructions; expression was normalized according to the $2^{-\Delta\Delta CT}$ method, and the highest scaling option was used (the sample with highest expression was assigned the value 1 in the relative mRNA calculation) (Kiselev et al. 2011a,b). The *Strongylocentrotus intermedius* actin (GenBank Accession Number DQ-229162) and ubiquitin (LOC754856) genes were used as endogenous controls to normalize variance in the quality and amount of cDNA used in each real-time RT-PCR experiment. A non-template control for each primer set and a non-RT control (DNase-treated RNA as a template) for each developmental stage were included. No-cycle threshold (Ct) values were consistently obtained after 50 cycles of PCR. The TaqMan probe for the actin and ubiquitin genes was labeled with a FAM reporter dye at the 5′-end and a RTQ-1 quencher dye at the 3′-end, and TaqMan probes for the *pks* and *sult* genes were labeled with an ROX reporter dye at the 5′-end and a BHQ-2 quencher dye at the 3′-end (Syntol). Data were summarized from 5 independent experiments. Primers and probes for the actin, *pks*, and *sult* genes were described previously (Ageenko et al. 2011). The primers 5′-AGC AGC GTC TCA TCT TCG-3′ and 5′-GAT GTT GTA GTC TGA GAG A-3′ and the probe 5′-TGC GGC CAT CCT CGA GTT GCT TGC CA-3′ were used for expression analysis of the ubiquitin gene.

Experiments with shikimic acid

Previously, we showed that the addition of ShA, which is a precursor of naphthoquinone pigments, to zygotes and embryos increased expression of the *pks* and *sult* genes; however, the addition of 2 mM ShA to zygotes, blastula, and gastrula embryos led to significant disturbances in embryonic development or embryonic growth arrest (Ageenko et al. 2011). Therefore, we used 0.5 mM of ShA for our experiments. Sterile solutions of ShA in seawater at the desired concentrations (0.5 mM) were added to sea urchin zygotes (after 20 min pf). Sea urchin zygotes without ShA were used as controls. Embryos were cultivated with ShA for 2 to 3 d. After this period, total RNA was isolated from the larvae for the quantitative real-time PCR as described previously (Ageenko et al. 2011). Three independent experiments were accomplished to examine the effect of bacteria and ShA on sea urchin development. ShA was obtained from Sigma. Experiments with bacteria and ShA were conducted as described in 'Materials and methods; Experiments with marine bacteria', except that we used sterile seawater with equal concentrations of ShA.

Statistical analysis

Statistical analysis was carried out using the Statistica 10 program. The results are represented as means (±SE) and were tested by paired Student's *t*-test; $p < 0.05$ was selected as the point of minimal statistical significance in all analyses.

RESULTS

Culturable bacteria associated with *Strongylocentrotus pallidus*

We isolated 22 bacterial strains from the cavity fluid of the sea urchin *Strongylocentrotus pallidus* from the Sea of Okhotsk and characterized these bacteria into the following 9 genera by phylogenetic analysis based on 16S rRNA gene sequencing: *Aliivibrio*, *Bizionia*, *Colwellia*, *Olleya*, *Paenibacillus*, *Photobacterium*, *Pseudoalteromonas*, *Shewanella*, and *Vibrio*. The most abundant isolates were within the genera *Vibrio* (36.4%) and *Aliivibrio* (18.2%). Approximately 9.1% of the obtained strains were from *Colwellia*, *Pseudoalteromonas*, and *Shewanella*, and approximately 4.5% of the strains were from *Bizionia*, *Olleya*, *Paenibacillus*, and *Photobacterium* (Table 2). We utilized a strain from each genus for further experiments, and these strains included the following: *Aliivibrio* sp. TH-K1, *Bizionia* sp. TH-K2, *Colwellia* sp. TH-K3, *Olleya* sp. TH-K4, *Paenibacillus* sp. TH-K5, *Photobacterium* sp. TH-K6, *Pseudoalteromonas* sp. TH-K7, *Vibrio* sp. TH-K8, and *Shewanella* sp. TH-K9.

The physiological, biochemical, and morphological characteristics of strains used are listed in Table 1.

Table 2. Diversity of bacterial strains isolated from the cavity fluid of *Strongylocentrotus pallidus* from the Sea of Okhotsk (22 strains). Percentage of strains in parentheses

Genus	Number of strains
Vibrio	8 (36.4)
Aliivibrio	4 (18.2)
Colwellia	2 (9.1)
Pseudoalteromonas	2 (9.1)
Shewanella	2 (9.1)
Bizionia	1 (4.5)
Olleya	1 (4.5)
Photobacterium	1 (4.5)
Paenibacillus	1 (4.5)

From the antibiotics tests, we determined that carbenicillin and erythromycin at concentrations of 100 and 15 µg ml^{-1}, respectively, were effective in inhibiting the growth of all tested bacteria (Table 1), with the exception of *Photobacterium* sp. TH-K6 for erythromycin. Ampicillin, benzylpenicillin, cephalexin, cephazolin, lincomycin, nalidixic acid, ofloxacim, polymyxin, rifampicin, streptomycin, tetracycline, and vancomycin were effective in inhibiting the growth of some of the tested bacteria, but gentamicin did not inhibit the growth of any of the bacteria (Table 1).

Influence of marine bacteria

The influence of bacteria on sea urchin development was repeated in 3 independent experiments, and results were similar. No apparent effect on normal embryonic development was detected after the addition of the following bacterial strains to sea urchin zygotes, which developed into morphologically nearly normal plutei (Fig. 1, Table 3): *Pseudoalteromonas* sp. TH-K7

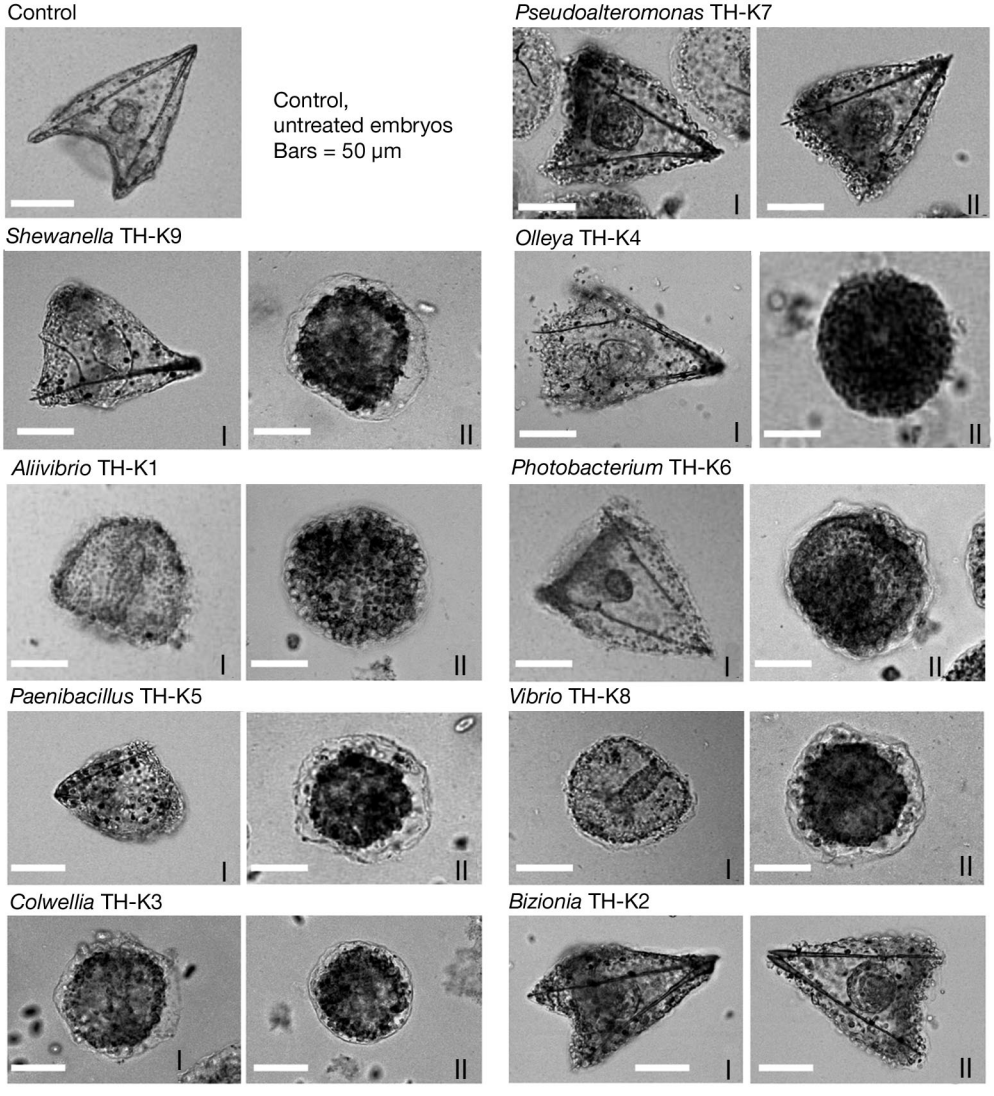


Fig. 1. Effect of cultured bacteria on larval morphology of the sea urchin *Strongylocentrotus intermedius*. I: 100 000 bacterial colony-forming units (CFU) ml^{-1}; II: 500 000 CFU ml^{-1}. Sea urchins were cultivated for 3 d

Table 3. Effect of cultured bacteria on larval morphology of the sea urchin *Strongylocentrotus intermedius* at 2 treatment doses: 100 000 colony-forming units (CFU) ml^{-1} (I) and 500 000 CFU ml^{-1} (II). Sea urchins were cultivated for 3 d. Data are means (±SEM) obtained from 3 independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$) versus values of the larvae of sea urchins that were not treated with marine bacteria

Bacterial strain	Treatment dose	Blastula stage (%)	Gastrula stage (%)	Prism stage (%)	Pluteus stage (%)
Control	0	0	0	1 ± 1	99 ± 1
Aliivibrio sp. TH-K1	I	0	93 ± 5**	7 ± 3	0**
	II	96 ± 8**	4 ± 3	0	0**
Bizionia sp. TH-K2	I	0	0	3 ± 2	97 ± 7
	II	0	0	4 ± 3	96 ± 5
Colwellia sp. TH-K3	I	21 ± 6*	79 ± 8**	0	0**
	II	72 ± 7*	28 ± 4**	0	0**
Olleya sp. TH-K4	I	0	0	4 ± 3	96 ± 9
	II	93 ± 11**	7 ± 6	0	0**
Paenibacillus sp. TH-K5	I	0	0	5 ± 4	95 ± 8
	II	93 ± 9**	7 ± 5	0	0**
Photobacterium sp. TH-K6	I	0	0	5 ± 4	95 ± 7
	II	4 ± 3	96 ± 6**	0	0**
Pseudoalteromonas sp. TH-K7	I	0	0	13 ± 6*	87 ± 7
	II	0	0	15 ± 7*	85 ± 8
Vibrio sp. TH-K8	I	4 ± 3	96 ± 7**	0	0**
	II	95 ± 5**	5 ± 4	0	0**
Shewanella sp. TH-K9	I	0	0	8 ± 3*	92 ± 9
	II	15 ± 4*	85 ± 7**	0	0**

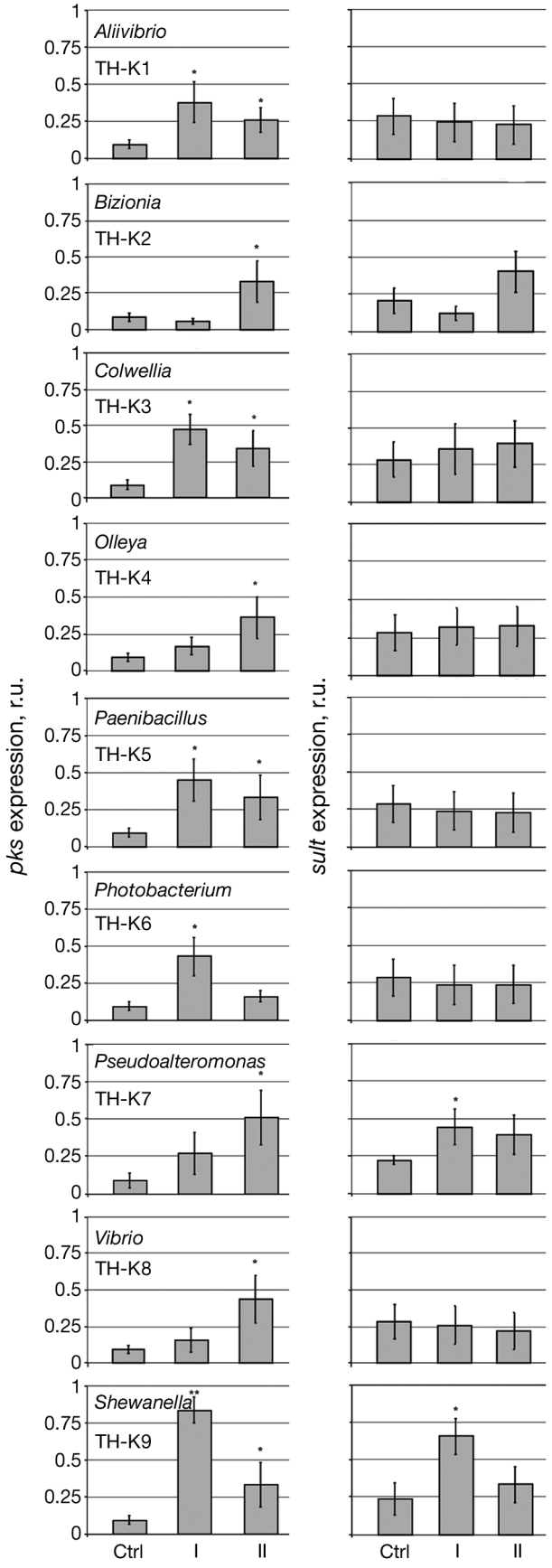
(100 000 and 500 000 CFU ml^{-1}), *Bizionia* sp. TH-K2 (100 000 and 500 000 CFU ml^{-1}), *Shewanella* sp. TH-K9 (100 000 CFU ml^{-1}), *Photobacterium* sp. TH-K6 (100 000 CFU ml^{-1}), *Paenibacillus* sp. TH-K5 (100 000 CFU ml^{-1}), and *Olleya* sp. TH-K4 (100 000 CFU ml^{-1}). Addition of the bacterial strains *Aliivibrio* sp. TH-K1 (100 000 CFU ml^{-1}), *Vibrio* sp. TH-K8 (100 000 CFU ml^{-1}), *Colwellia* sp. TH-K3 (100 000 CFU ml^{-1}), *Shewanella* sp. TH-K9 (500 000 CFU ml^{-1}), and *Photobacterium* sp. TH-K6 (500 000 CFU ml^{-1}) to sea urchin zygotes resulted in a slowing of embryo development. After 3 d of cultivation with these bacterial strains, the development of >79% of the sea urchin larvae was suppressed in the gastrula stage (Fig. 1, Table 3). Furthermore, the addition of high concentrations (500000 CFU ml^{-1}) of *Aliivibrio* sp. TH-K1, *Paenibacillus* sp. TH-K5, *Colwellia* sp. TH-K3, *Vibrio* sp. TH-K8, and *Olleya* sp. TH-K4 to the zygotes caused significant disturbances in embryonic development, with 72 to 96% of the analyzed embryos remaining spherical in shape or in growth arrest (Fig. 1). In addition, the amount of pigment cells increased after the treatment of the larvae with bacteria, especially with *Pseudoalteromonas* sp. TH-K7 and *Bizionia* sp. TH-K2 (data not shown). This increase in the amount of pigment cells correlated with an increase in viability of the sea urchin embryos.

We analyzed expression of the *pks* and *sult* genes in sea urchin larvae after treatment with bacteria. We showed that all 9 strains significantly increased *pks* expression (Fig. 2). The highest *pks* expression was detected after incubation with *Shewanella* sp. TH-K9, which increased *pks* expression 9.1-fold compared to untreated cells (Fig. 2). Two strains of the utilized bacteria (*Pseudoalteromonas* sp. TH-K7 and *Shewanella* sp. TH-K9) significantly increased *sult* expression by 2- to 3-fold compared to untreated cells (Fig. 2).

Influence of shikimic acid

When first using ShA, we verified the influence of 0.5 mM ShA on the viability of the utilized strains of bacteria. We showed that 0.5 mM of ShA did not affect the viability of any of the strains used (data not shown); therefore, we performed experiments using *Strongylocentrotus intermedius* larvae. The influence of the isolated bacteria and ShA on sea urchin development was repeated in 3 independent experiments, and results were similar.

In experiments with ShA, we used bacteria of the *Colwellia* sp. TH-K3 and *Vibrio* sp. TH-K8 strains, because these strains were found to be more pathogenic to sea urchin embryos in our previous experiments (Fig. 1). We also used *Olleya* sp. TH-K4 as an example of the strains that are pathogenic at high concentration (500 000 CFU ml^{-1}). Addition of ShA simultaneously with bacteria (100 000 CFU ml^{-1}) increased the viability of sea urchins and normalized



their development (Fig. 3). Additionally, we detected an increase in the number of pigment cells and *pks* and *sult* expression (Fig. 4), as has previously been described (Ageenko et al. 2011). Expression of *pks* and *sult* genes increased 1.2- to 6.3- and 1.2- to 2.4-fold compared to untreated larvae, respectively (Fig. 4). However, even with the addition of ShA, the *Colwellia* sp. TH-K3, *Vibrio* sp. TH-K8, and *Olleya* sp. TH-K4 strains, when in concentrations of 500 000 CFU ml^{-1}, were still pathogenic and suppressed sea urchin development and viability (98 to 100% of analyzed embryos remained spherical in shape or in growth arrest; Fig. 3).

DISCUSSION

We isolated 22 bacterial strains from the cavity fluid of the sea urchin *Strongylocentrotus pallidus* from the Sea of Okhotsk, a close congeneric species of *S. intermedius*. Then, we separated the bacteria into the following 9 genera by phylogenetic analysis based on 16S rRNA gene sequences: *Aliivibrio*, *Bizionia*, *Colwellia*, *Olleya*, *Paenibacillus*, *Photobacterium*, *Pseudoalteromonas*, *Shewanella*, and *Vibrio*. It should be noted that our generic composition of the normal bacterial community of the sea urchin *S. pallidus* differed from the composition that was described previously for *Hemicentrotus pulcherrimus* from the South China Sea (Huang et al. 2009). In this report, we first described the bacterial composition of sea urchins from the Sea of Okhotsk. Furthermore, we investigated the influence of 9 strains, from each of the different genera that were identified, on larvae viability, the amount of pigment cells, and the *pks* and *sult* expression.

Many publications have been reported regarding the pathogenicity of species of *Pseudoalteromonas*, *Shewanella*, and *Vibrio* for echinoderms (Gatesoupe et al. 1999, Molini et al. 2008, Becker et al. 2009, Deng et al. 2009, Li et al. 2010). Many species of *Vibrio* are responsible for diseases in marine organisms and economic losses to the aquaculture industry

Fig. 2. Levels of *pks* and *sult* expression detected by real-time PCR from larvae of the sea urchin *Strongylocentrotus intermedius* that had been treated with bacteria. Ctrl: untreated larvae; I: 100 000 bacterial CFU ml^{-1}; II: 500 000 bacterial CFU ml^{-1}; r.u.: relative units. Sea urchins were cultivated for 3 d. Data are means (±SEM) obtained from 3 independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$) versus values of larvae of sea urchins that were not treated with marine bacteria

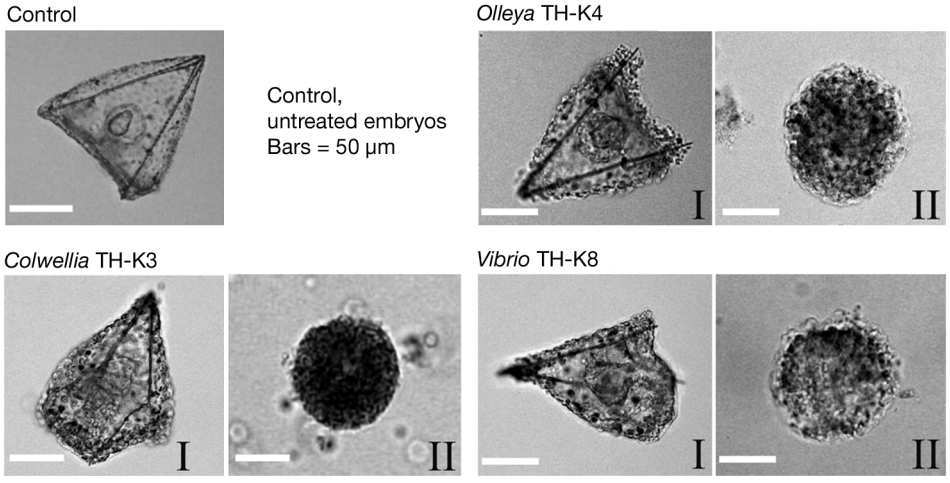


Fig. 3. Effect of cultured bacteria, with the addition of shikimic acid, on larval morphology of the sea urchin *Strongylocentrotus intermedius*. I: 100 000 bacterial colony-forming units (CFU) ml^{-1}; II: 500 000 CFU ml^{-1}. Sea urchins were cultivated for 3 d

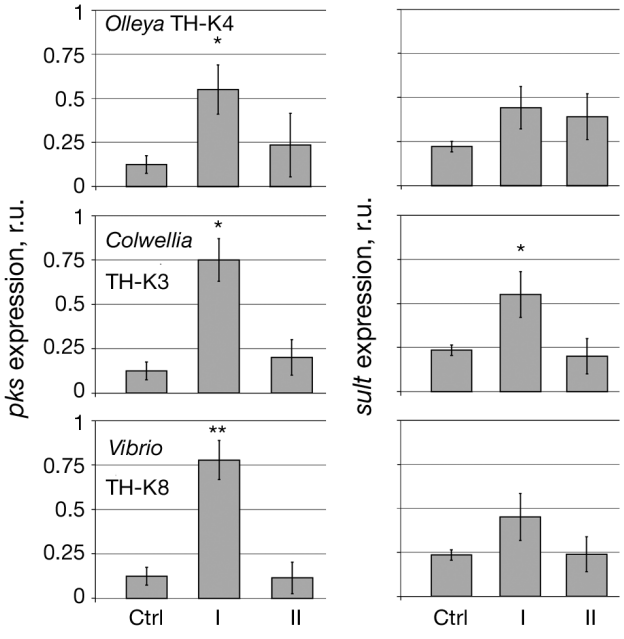


Fig. 4. Levels of *pks* and *sult* expression detected by real-time PCR from larvae of the sea urchin *Strongylocentrotus intermedius* that had been treated with bacteria, with the addition of shikimic acid (ShA). Ctrl: untreated larvae; I: 100 000 bacterial CFU ml^{-1}; II: 500 000 bacterial CFU ml^{-1}; r.u.: relative units. Sea urchins were cultivated for 3 d. Data are means (±SEM) obtained from 3 independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$) versus values of larvae of sea urchins that were not treated with marine bacteria or ShA

(Gay et al. 2004, Molini et al. 2008). *Vibrio* sp., *V. splendidus*, and *V. harveyi* have been reported to be major, well-known pathogenic bacteria (Gatesoupe et al. 1999) and are known to be the pathogens that cause the skin ulceration syndrome of the sea cucumber *Apostichopus japonicus* (Deng et al. 2009, Li et al. 2010). Since sea urchins and sea cucumbers are closely related classes, it is not surprising that *V. splendidus*, *V. harveyi*, and 2 other similar species, *V. shilonii* and *V. fortis*, were also found to cause some diseases in sea urchins (Wang et al. 2012). Additionally, we showed that *Vibrio* sp. TH-K8, which was most closely related to *V. kanaloae* by 16S rRNA gene sequences, was pathogenic to *Strongylocentrotus intermedius* larvae. The pathogenicity of *V. kanaloae* for mollusks has previously been reported (Guisande et al. 2008), but we suggest that *Vibrio* sp. TH-K8 is pathogenic for echinoderms. For a more precise identification of the *Vibrio* spp. TH-K8, sequence analysis of the *topA-mreB* gene is necessary (Rivera-Posada et al. 2011).

Shewanella aquimarina has also been shown to be associated with the lesion syndrome of *Strongylocentrotus intermedius* (Wang et al. 2012), and the first report of *S. aquimarina* in seawater was for strains that were isolated from the Yellow Sea in Korea (Yoon et al. 2004). However, it is well known that the genus *Shewanella* is widely distributed in marine environments and that some members of the genus, such as *S. algae* and *S. putrefaciens*, are opportunistic human pathogens (Pagani et al. 2003). It has been suggested that aquatic animals might be the natural reservoirs of this human pathogen (Beleneva et al. 2009), and our results and data from Wang et al. (2012) suggested that the genus *Shewanella* might be another prominent pathogen of echinoderms.

Pseudoalteromonas tetraodonis is common in marine waters and is generally associated with various microorganisms. This organism causes skin ulcerations in *Apostichopus japonicus* (Li et al. 2010) and might cause disease in the sea urchins *Meoma ventricosa* and *Strongylocentrotus intermedius* (Nagelkerken et al. 1999, Wang et al. 2012). However, in our

experiments, the amount of *Pseudoalteromonas* sp. TH-K7 that was used was not pathogenic for *S. intermedius* larvae. It was found that the normal bacterial community of the sea urchin *Hemicentrotus pulcherrimus* included some bacteria from the genus *Pseudoalteromonas* (Huang et al. 2009). Our results showed that the normal bacterial community of the sea urchin *S. intermedius* also included some bacteria from the genus *Pseudoalteromonas*; therefore, we suggested that members of the genus *Pseudoalteromonas* are opportunistic pathogens of sea urchins.

It is known that species of *Aliivibrio*, *Photobacterium*, and *Paenibacillus* are pathogens of some fishes and insects (Osorio et al. 2000, Yokoyama et al. 2003, Urbanczyk et al. 2007). Our results indicated that the species from these genera were pathogenic for *Strongylocentrotus intermedius* larvae; however, the neither the pathogenicity of *Colwellia* nor of *Olleya* was known. Our data suppose a strong pathogenicity of *Colwellia* and, to a lesser extent, that of *Olleya*. Therefore, we suggest that the members of the *Colwellia* and *Olleya* genera are opportunistic pathogens of sea urchins.

During our research, we discovered that the cavity fluid of apparently healthy *Strongylocentrotus pallidus* sea urchins contained several pathogenic bacteria. It is possible that the samples were unintentionally contaminated during the collection process or that at least some of the animals were in the asymptomatic stages of an infection. Nevertheless, it is well known that bacterial infections cause major disease problems, with associated high mortalities among juvenile sea urchins in large aquaculture facilities, whereas adult sea urchins appear resistant to significant losses from diseases (Böttger et al. 2004). We propose that mature sea urchins have an increased immunity against the bacteria compared to the susceptibility of larvae. Perhaps, high concentrations of the polyketide compounds help mature sea urchins combat bacterial diseases.

In the second part of our study, we searched for the mechanisms of resistance that are used against the tested strains of bacteria. The usual method of resistance is the use of antibiotics; however, activation of the biosynthesis of antibacterial pigments in sea urchins also occurs. Earlier Böttger et al. (2004) showed that using gentamycin (10 mg l^{-1}) and neomycin (30 mg l^{-1}) is effective in inhibiting bacterial growth in the sea urchin aquaculture, but in our experiments gentamycin did not inhibit growth of the tested bacteria and neomycin was effective only for half the used strains. After testing the efficiency of different antibiotics, we determined that carbenicillin and erythromycin at the concentrations of 100 and 15 mg ml^{-1}, respectively, were effective in inhibiting the growth of all tested bacteria strains (with the exception of *Photobacterium* sp. TH-K6 for erythromycin). It is possible that treatment of mass quantities of sea urchin larvae in aquaculture with antibiotics was not effective because unsuitable antibiotics were used.

We showed that the amount of pigment cells increased after the incubation of sea urchin larvae with bacterial cells. Pigment cells are the first type of second mesenchymal cells (Ruffins & Ettensohn 1993). These cells accumulate red-brown pigment granules in their cytoplasm (Chaffee & Mazia 1963), and these granules store carotenoids and naphthoquinone compounds (Ryberg & Lundgren 1979), which have been suggested to function in body coloring and phototropism and to aid in the defense of the larval ectoderm (Matsuno & Tsushima 2001, Smith et al. 2006). Pigment cell precursors are released from the vegetal plate during the initial phase of gastrulation, and these precursors have the ability to migrate within the ectodermal layer of the larval epithelium (Gibson & Burke 1985). The ability to phagocytose, which is exhibited by pigment cells, suggests that these cells participate in wound healing in larvae (Hibino et al. 2006). Additionally, our data indicate the probable involvement of pigment cells in the defense response against marine bacteria.

In addition to the increasing amount of pigment cells, we detected a significant increase in expression of the *pks* and *sult* genes, which are responsible for the biosynthesis of polyketide compounds. Furthermore, treatment with ShA activated the molecular signaling pathways that are involved in the biosynthesis of pigments during sea urchin development, and, consequently, we detected an increased immunity against bacteria of treated sea urchin larvae. The gained effect could not be attributed to the antimicrobial activity of ShA, because, in additional experiments, ShA did not display this activity. Therefore, we suggest that activating biosynthesis of naphthoquinone pigments by feeding biosynthetic precursors of pigments to sea urchin embryos may be a new technique to protect the sea urchin larvae from bacterial infection in aquaculture. It is possible that the precursors of naphthoquinone pigments display a wide range of antimicrobial activities which allow them to protect larvae from bacterial infection. Thus, some of the results presented here might contribute to the development of new techniques for sea urchin aquaculture.

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