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ORIGINAL RESEARCH

Specific antibacterial activity against potential pathogens and re-straining of larvae settlement from a pigmented *Pseudoalteromonas* strain isolated from the jellyfish *Cassiopea xamachana*

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Abstract The bacterial genus *Pseudoalteromonas* is commonly found in the ocean and frequently associated with marine invertebrates. This bacterial group produces metabolites with antibacterial, algicidal, antifouling, and biofilm promoting activities that have interested the scientific community for years. Unlike corals, reports on diseased symbiotic, soft-body cnidarians are scarce, suggesting their surface mucus layer may be efficient in fighting potential bacterial pathogens. With the aim to study attributes of cnidaria-associated bacteria, we isolated a *Pseudoalteromonas* pigmented strain from the surface mucus layer of a bleached jellyfish *Cassiopea xamachana* with a 99,66% similarity to *P. piscicida*, as identified through 16S rRNA sequencing. This *Pseudoalteromonas* sp. yellow isolate specifically inhibited the growth of the potential bacterial pathogen *Serratia marcescens,* and an *Aurantimonas* sp. strain. This isolated *Pseudoalteromonas* strain also showed activity against epibionts, reducing the settlement of medusa larvae by 50% compared to controls. A 40% inhibition in the settlement of medusa larvae was scored for an organic extract prepared from a filtrate of the cultured isolate as well as positive protease activity. Overall, our results suggest *Pseudoalteromonas* sp. yellow isolate can fight potential pathogens and produce extracellular vesicles with metabolites that can influence the settlement and survival of larvae, presenting positive attributes for this animal host.

Keywords Commensal bacteria . Larval settlement . Extracellular vesicles . Antimicrobial . Epibionts

Introduction

The surface mucus layer (SML) that covers zooxanthellate-cnidarians serves as an immune barrier that protects their live tissues from pathogens and other external threats (Brown and Bythel 2005). Due to its composition rich in carbohydrates and proteins, the SML hosts a diverse set of microorganisms that shape the intrinsic microbiome of these cnidarians (Paul et al. 1986; Hayes and Goreau 1998; Sutherland et al. 2004; Krediet et al. 2013). Some of these bacteria exhibit receptors that can bind to the mucus layer, suggesting the SML may contribute to structuring its own microbiota (Rosenberg and Falkovitz 2004; Zaragoza et al. 2014) and all together, sustain a healthy microbial community (Ritchie 2006; Mao-Jones et al. 2010). For instance, the coral *Acropora palmata* hosts bacteria that block the induction of glycosidases in the bacterial pathogen *Serratia marcescens*, lowering its virulence and preventing its ability to grow in the SML (Krediet et al. 2009; Krediet et al. 2012). However, under environmental deteriorating conditions,

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some opportunistic pathogens may grow, destabilize, and outcompete healthy residing microorganisms, sickening the hosts (Rublee et al. 1980; Toren et al. 1998; Sekar et al. 2006; Harvell et al. 2007).

Bacteria that reside in the SML, and other marine biofilms, produce different bioactive molecules that can fight other bacteria, have antifungal or algicidal roles, and inhibit or favor the settlement of invertebrate larvae. Such bioactive molecules include toxic proteins, pyrrole-containing alkaloids, cyclic peptides, or polyanionic exopolymers, with various potential roles (Bowman 2007). Among bacteria that belong to marine microbiomes are pigmented species of the genus *Pseudoalteromonas* which are present in biofilms associated with crustose coralline algae (CCA). Bacteria isolated from this biofilm produce a tetrabromopyrrole that induces the settlement of coral larvae (Sneed et al. 2014). Additionally, other compounds isolated from CCA may also induce the metamorphosis of larvae in Pacific corals (Tran and Hadfield 2011); however, this also occurs in the absence of CCA (Webster et al. 2004). Commensal bacteria associated with the SML can produce antimicrobial compounds that protect corals (Ritchie 2006; Nissimov and Munn 2009; Shnit-Orland and Kushmaro 2009); although bacteria with antimicrobial activities in coral SML are found only after perturbations that may lead to disease (Kvennefors et al. 2012).

Soft body symbiotic cnidarians, like the medusa *Cassiopea xamachana* and the anemone *Exaiptasia pallida (diaphana)*, both model organisms for studies in symbiosis (Sunagawa et al. 2009; Ohdera et al. 2018), may lose their algal symbionts under high-temperature stress and bleach. Although some reports comment on occasional unhealthy conditions for the animals kept in captivity (Wang et al. 2008; personal observations), there are no reports of these cnidarians getting diseased, probably because they soon die and disintegrate. However, their bacterial community and SML protection may be effective at fighting potential pathogens. Coral disease susceptibility was suggested to be associated with changes in the composition of the SML, the loss of antibiotic activity of resident microbes, and the increase in pathogenic ones (Mao-Jones et al. 2010). Also, some bacteria inhabiting the SML may produce compounds that can disrupt the progression of a disease, as was experimentally shown in *E. pallida (diaphana)* infected with *S. marcescens* (Alagely et al. 2011). Experimental infections of this anemone with coral pathogens like *Vibrio coralliilyticus* and *V. shiloi*, *S. marcescens*, and a consortium of bacteria recovered from yellow band diseased corals, produced darkening of the tissues, retraction of tentacles, and disintegration of the polyps (Krediet et al. 2012; Zaragoza et al. 2014; Brown and Rodríguez-Lanetty 2015).

Pseudoalteromonas is a common, gram-negative bacterial genus found in the sea. Pigmented and non-pigmented strains correlate with the ability to produce bioactive compounds with a diversity of properties (Lemos et al. 1985; Egan et al. 2002; Holmström et al. 2002; Bowman 2007). Also, some species influence the formation of biofilms and induce the settlement and metamorphosis of invertebrate larvae (Bowman 2007; Whalen et al. 2015). However, some *Pseudoalteromonas* species have been found to reduce larval settlement and metamorphosis of marine invertebrates (Huang et al. 2011; Zeng et al. 2017). *Pseudoalteromonas* are also reported as epiphytes of brown and green algae, with antibiotic-producing capabilities (Lemos et al. 1985). Since *Pseudoalteromonas* are frequently associated with eukaryotic hosts (Holmström and Kjelleberg 1999), they may play an ecological role, defending their hosts from potential pathogenic bacteria and encrusting organisms (Shnit-Orland and Kushmaro 2009).

Pseudoalteromonas is also commonly associated with corals (Rohwer et al. 2002; Wegley et al. 2007; Shnit-Orland and Kushmaro 2009) as an abundant group of culturable bacteria (Shnit-Orland and Kushmaro 2009; Kelly et al. 2014). But a *Pseudoalteromonas* strain was found to associate with the coral disease known as yellow band (Cervino et al. 2004). In contrast, various bacterial pathogens that have been related with diseased corals, like *Serratia marcescens, Vibrio shiloi, V. coralliilyticus* and *Thalassomonas loyana*, are effectively neutralized by some *Pseudoalteromonas* species (Sneed et al. 2014). Considering that closely related strains may produce various metabolites with different activities, the identity of each strain becomes relevant (Offret et al. 2016). Further, the high production and diversity of extracellular proteases in the *Pseudoalteromonas* bacterial group have attracted interest for their potential application and ecological roles, mainly in nitrogen degradation and their interaction with other bacteria (Chen et al. 2020).

We are interested in studying the antimicrobial potential and ecological attributes of bacteria associated with the SML of cnidarians (Rivera-Ortega and Thomé 2018). Here, we report on a partial characterization of an isolated bacterium from the SML of *C. xamachana* jellyfish. In addition to studying the antibacterial potential of the isolated bacterium, we have used the jellyfish larvae as proxies for the capacity of the

bacterium to inhibit larval settlement, since the larvae from this jellyfish can be produced with relative ease.

Materials and methods

Isolation of bacterial strains

Cassiopea xamachana medusae were originally donated by the Xcaret Park and maintained in our seawater tank facilities for several years. To bleach *C. xamachana* we followed the cold shock protocol proposed by Estes et al. (2003) and Muscatine et al. (1991). We placed medusae into filtered sea water at 5°C ± 1°C for 4 h. After the cold shock medusae were placed in a dark aquarium during 2 to 3 weeks until they reached a visual pallid tissue color. The yellow-colored bacterial colonies were isolated from bleached medusas. To isolate the yellow-colored bacterial colonies we plated mucus samples and consecutively re-plated (x 3) the bacteria on Marine Agar (Sobel Marine Agar, DIFCO) assaying for their antimicrobial potential. A yellow isolate (YI) was further cultured on Marine Agar at 27°C for its identification and partial characterization.

Antimicrobial assays over tester strains

We tested the capacity of the YI bacteria to inhibit the growth of three tester bacterial strains. Tester strain *Serratia marcescens* (ATCC, strain BAA-632, isolated from coral) has been described as a coral pathogen (Patterson et al. 2002); we also used an *Aurantimonas* sp. (ATCC, strain BAA-667, isolated from seawater) as an approximation, since this genus has been associated with diseased corals in the Caribbean (Denner et al. 2003). In addition, we used the non-pathogenic *Escherichia coli* commercial strain JM109 (Promega). Tester bacteria were cultured in Marine Broth overnight at 27°C with shaking (120 rpm). The potential growth inhibition of the isolated bacteria was assessed by a swarming combined with the double agar overlay assay, following Rivera-Ortega and Thomé (2018). Briefly, sterile filter paper discs (8 mm. diameter) embedded in 7 µL of an overnight culture of the isolated *Pseudoalteromonas* sp., were layered onto hard Marine Agar (40 % agar). Next, we poured a layer of soft agar over (6 % agar), mixed with 100 µL of an overnight culture of the tester strains. The plates were incubated at 27°C for 24 hours for the growth of *S. marcescens* and *E. coli,* and 48 hours for *Aurantimonas* sp. and scored for the presence of a growth inhibition area around the discs. We further tested the capacity of the *Pseudoalteromonas* sp. YI to affect the growth of the yeasts *Saccharomyces cerevisiae* (strain W303) and *Debaryomyces hansenii* (strain Y4728). For this, we used Marine Agar mixed with 100 µL of each yeast culture and layered with filter discs embedded in 10 µL of a YI overnight culture. The plates were then scored for a growth inhibition area after 2 days of culturing at 27°C in an environmental chamber.

DNA extraction and manipulation

DNA was extracted from cultures of the isolated bacteria in Marine Agar, grown in an environmental chamber at 27°C. Cells were collected with a scraper, resuspended in Marine Broth (DIFCO) and sedimented by centrifugation. The DNA was extracted with a Genomic DNA purification kit (Promega, USA) and its integrity checked in agarose gels. We used two PCR amplifications to sequence a fragment of 1496 nucleotides total for the 16S rDNA, using primers 27F + 806R and primers 515F + 1492R. The PCR reactions were performed with 1µL of DNA, 3 mM MgCl2 and 3 U of DreamTaq© DNA polymerase. Amplicons were cloned into the pDRIVE vector (QIAGEN) and sequenced at the Molecular Biology Facility at IFC-UNAM and aligned. The 1496 bp long sequence was compared by BLAST to the nt collection and Refseq databases for identification. The identity of all sequences was also checked at the EZBioCloud database. The sequence for the *Pseudoalteromonas* sp. yellow isolate was submitted to GenBank with accession number MZ493910.

Isolation of *Cassiopea xamachana* larvae

Asexual, apo-symbiotic larvae (buds) were collected from *C. xamachana* scyphistomae (polyps) that we

maintain in our laboratory and naturally produce them (Colley and Trench 1983). The polyps are maintained at room temperature, in the dark, to avoid accidental infection with potential dinoflagellate symbionts. The polyps are fed once a week with freshly hatched *Artemia* nauplii, with a change of filtered seawater the day after. Asexual larvae were collected one week after abundant feeding of polyps (every other day), by filtering through 10 µm filters. We used buds that were not yet differentiated. The larvae settlement experiments were set 1 to 3 days after their collection.

Settlement assays of asexual larvae

We used multi-well plastic plates (with 6 wells) for the experiments. The plates were pretreated to ensure they were bacteria-free by soaking in 10 % hypochlorite for 1 hour and extensively rinsed in purified water. For control plates, each well was filled with 5 mL of sterile artificial seawater (ASW) adding two larval buds per well for a total of six wells, and incubating for 7-9 days in the dark at 27°C. For experimental plates in the lawn assay, each well was filled with 5 mL of Marine Broth. We inoculated each well with 50 µL of an overnight bacterial culture (either *Pseudoalteromonas* sp. Yellow Isolate, *Serratia marcescens* BBA-632, *Aurantimonas* sp. BBA-667, or *Escherichia coli* JM101) and incubated for 3 days at 27°C in an environmental chamber to allow for a bacterial lawn to grow. The culture medium was then carefully removed and replaced with sterile ASW. Two larval buds were added to each one of six wells and kept in the dark at 27°C for 7 days when observations were recorded under a stereoscopic microscope. The ASW in all plates was carefully replaced once after 4 days. The percentage of settled larvae was compared to control settlement on bacteria-free plates. Experiments were repeated at least three times. For settlement assays with organic extracts, we used filtered-sterilized seawater (FSW) recording settlement after 7-9 days.

Preparation of organic extracts

We followed the protocol by Sneed and collaborators (2014) to prepare organic crude extracts. Briefly, the yellow bacterial strain was cultured on Marine Agar plates at 27°C until a thick lawn had grown (48h). The bacteria scraped from the surface of each plate were lyophilized and stored at -20°C. Lyophilized bacteria were extracted in 4 mL of methyl acetate:methanol (1:1) per plate, allowing solvents to evaporate in a fume hood for 24 hrs. The crude extract was dissolved in 1 mL of diluted DMSO or ethanol (0.02%, v/v) per plate, and clarified by centrifugation at 16,000 x g for 5 min. Each well of a multi-well plastic plate was filled with 5 mL of FSW, adding 50 µL or 100 µL of the organic extract to test the effect over the settlement of larval buds. The experiments were repeated two to three times and compared to controls with DMSO or ethanol only (using the same volume as the extract) or without organic solvents, after 8 days.

Assays with bacterial released substances

A second set of assays was performed to evaluate if inhibitory substances are being released by the *Pseudoalteromonas* sp. YI. The YI strain was grown in Marine broth overnight at 27°C. The culture was centrifuged to remove cells, filtered through 0.22 µm membranes and directly added to larvae. Each well of a multi-well plastic plate was filled with 5 mL of FSW, adding 50 µL or 100 µL of the organic extract to test the effect over the settlement of larval buds. This assay was repeated with a different setup to test the possible release of extracellular vesicles: the yellow strain was cultured on Marine Agar plates at 27°C until a thick lawn had grown (48h). The bacteria were scraped from the surface of each plate and resuspended in 2 mL of Marine Broth, vortexed, and filtered through 0.45 µm membranes. The filtrate was used directly in larval settlement assays as before.

Protease activity assays

We also evaluated the YI for protease activity with a general assay. The presence of serine proteases was determined as the increase in fluorescence in the filtrate of cultured yellow strain, prepared as above (cultured in Marine Agar plates). We used a Protease Fluorescent Detection Kit (SIGMA, USA) with FITC-

Table 1 Growth inhibition of bacterial tester strains and yeast strains by the isolated *Pseudoalteromonas* sp. yellow bacterium

	Serratia marcescens	*Aurantimonas sp.*	*Escherichia coli*	*Debaryomyces hansenii*	*Saccharomyces cerevisiae*
Assays	3/3	3/3	0/3	0/2	0/2

The numbers in each quotient: numerator, positive inhibition; denominator, number of independent experiments.

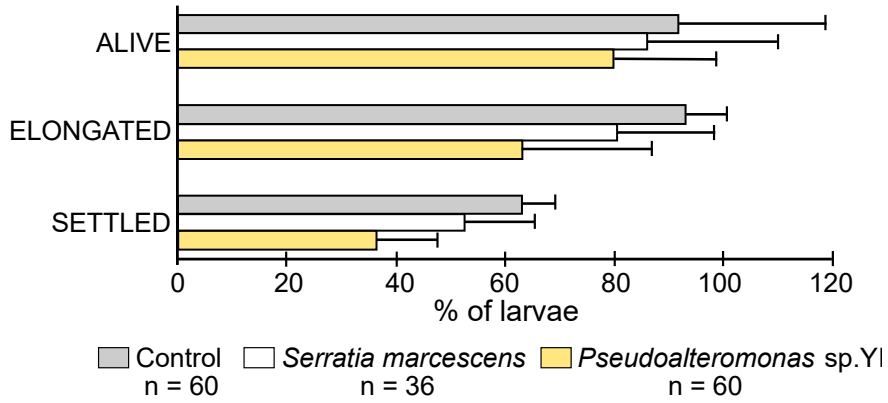

Fig. 1 Settlement and condition of *Cassiopea xamachana* asexual larvae incubated with a lawn of bacteria. *Pseudoalteromonas* sp. YI was isolated from the mucus of adult medusa. The vertical axis depicts categories on the observation of the larvae after 7 days of incubation at 27°C, incubated in the presence or absence (control) of bacteria. Data are the average of 3 to 5 independent experiments (12 larvae per experiment) for each strain ± SEM Total larvae used indicated below each treatment.

labeled casein as substrate. Results are reported as relative fluorescence units, normalized to the activity equivalent to 50 ng μL^{-1} of trypsin and to protein content. Soluble protein was estimated from centrifuged filtrates (14,000 rpm for 1 min) using the Bradford method with BSA as the standard. Readings of raw fluorescence were recorded 10 seconds after diluting 1:100 the FITC-labeled casein and bacterial filtrate with assay buffer, at room temperature.

Statistical analysis

For antibacterial assays, we applied the exact Fisher test to compare differences in the inhibition of the isolated bacteria upon tester strains. Assays for the settlement of larvae were analyzed with Chi-squared and Kruskal-Wallis tests due to the non-normal distribution of the data and the homogeneity of the variances (Tables 2 and 3). Normality was evaluated with a graphical test and homoscedasticity was determined by Killeen test (data not shown). All statistical analyses were performed in R software, version 3.5.2.

Results

Identification of isolated bacteria

We amplified two consecutive overlapping fragments of the 16S rDNA that, after alignment, produced a 1496 nt long sequence (Additional File_1). The YI was identified as belonging to the genus *Pseudoalteromonas*. A BLAST comparison to the GeneBank nt collection database indicated that the closest neighbor species known for the YI is *P. piscicida* strains DE2-A and DE2-B, being 99.66% similar [accession CP031761.1 and CP021646.1, respectively] with 5 nucleotides difference.

Antibacterial assays of isolated bacteria over tester strains

The *Pseudoalteromonas* YI strain inhibited the growth of tester strains *Serratia marcescens* and *Aurantimonas* sp. but did not have an effect against the growth of *Escherichia coli* or the yeasts *Saccharomyces cerevisiae* and *Debaryomyces hansenii* (Table 1), suggesting a rather specific effect.

Table 2 Kruskal-Wallis test for larval settlement experiments

Condition of larvae	Lawn assay (df= 2)	DMSO Extract (df=4)	ETOH Extract (df=4)
Alive	P = 0.24	P = 0.21	P = 0.87
Settled	P = 0.25	P = 0.08	P = 0.23
Elongated larvae	P = 0.05	P = 0.15	P = 0.69

df = degrees of freedom; P = P-value. Statistically significant P-values in bold.

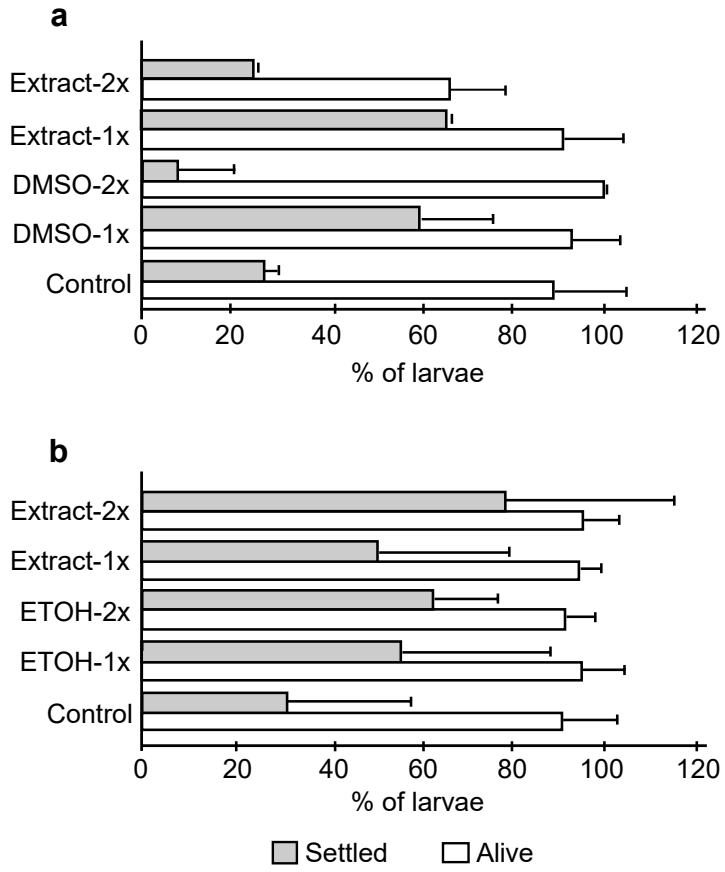

Fig. 2 Effect of an organic extract from *Pseudoalteromonas* sp. YI over the settlement of *Cassiopea xamachana* larvae. Organic extract dissolved in (a) DMSO or (b) ethanol, used at 1X (50 µL) or 2X (100 µL) final concentration. Controls for solvents contained FSW with organic solvents (0.02%, v/v). Overall controls had no additions. The survival and settlement of larvae were scored after 9 days of incubation at 27°C. Data are the average of one to three independent experiments (12 larvae per experiment) for each condition ± s.e.m. Total number of experiments in (a): Control (FSW) = 2.5; DMSO-1x (0.02%, v/v) = 1, Extract-1x (50 µL) = 1; DMSO-2x = 2, Extract 2x = 2. Total number of experiments in (b): Control (FSW) = 2.5; ETOH-1x (0.02%, v/v) = 2, ETOH-2x = 2, Extract-1x (50 µL) = 3.5; Extract-2x = 2.

Evaluating the settlement of larvae

To evaluate the capacity of *Pseudoalteromonas* sp. YI strain to affect the settlement of larvae, we exposed 12 larvae per experiment to a lawn of this strain and examined their behavior. The observations were scored after one week, which is the time the larvae usually take to settle onto plastic plates. We observed that the YI inhibited the settlement of larvae, allowing only 37% of larvae to settle, on average, compared to 63% settlement observed in controls (Fig. 1). To define if this effect was specific for the YI or general for bacteria, we also assayed the settlement of larvae with a lawn of *Serratia marcescens*, *Aurantimonas* sp., and *Escherichia coli*.

Aurantimonas sp. and *E. coli* resulted in high mortality of the larvae after 5 days (> 95% mortality, data not shown). In contrast, *S. marcescens* showed no inhibitory effect over the settlement of the larvae. A Kruskal-Wallis test indicated no statistical differences among treatments for the settlement and survival of larvae; however, there was a significant difference in the development of larvae, being lower than the control in the presence of the YI (Table 2).

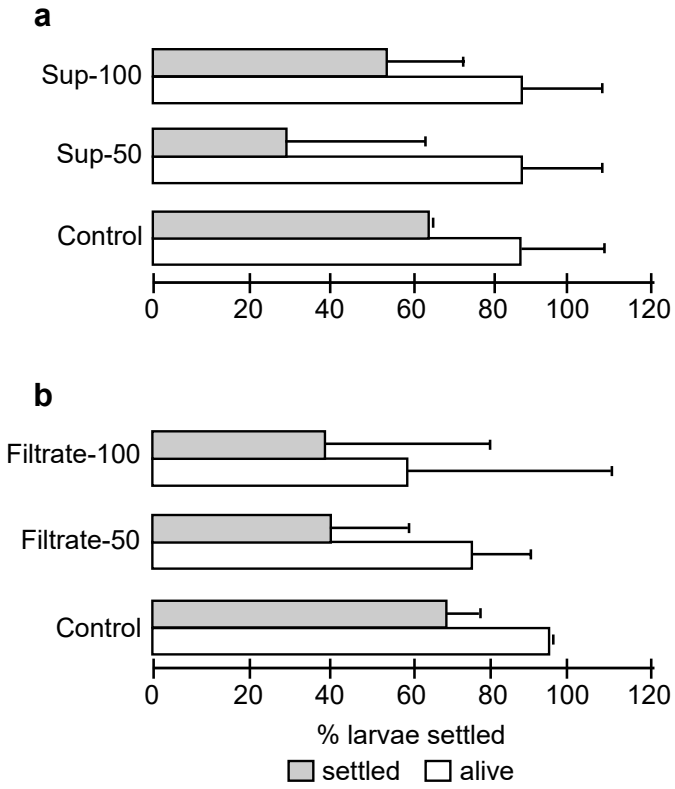

Fig. 3 Effect of substances released by *Pseudoalteromonas* sp. YI on larval settlement. Larvae were exposed to (a) supernatant of a liquid culture, collected after centrifugation, or (b) filtrate of a plate culture collected by filtration through 0.45 µm. Numbers in the vertical axis represent the volume of the extract added (50 or 100 µL). Results are the average of three independent experiments ± s.e.m. for a total of 9 (a) and 16 larvae (b) after 7 days of incubation.

The effect of organic extracts from *Pseudoalteromonas* sp. YI

Given that the effect of the tested bacteria on the settlement of larvae was highly variable and the *Pseudoalteromonas* sp. YI suggested some effect (Fig. 1), we prepared and tested an organic extract of the YI on the survival and settlement of larvae. The organic extract had no significant effect on the settlement and survival of the larvae when used at 1 X final concentration (Table 2; Fig. 2a). Increasing the amount of crude extract 2-fold (100 µL) reduced both the survival and settlement of the larvae. However, a strong effect of DMSO at 2-fold (2 %, v/v) was also observed, making it difficult to ascertain the effect of the organic extract over the settlement of larvae. To discern the effect of the organic compounds extracted from the YI bacterium, we used ethanol as a solvent and repeated the assays at the same final concentrations. Results showed no effect on the survival of larvae in all tested concentrations of either ethanol only, or ethanol-dissolved extract (Fig. 2b). Although results were not statistically significant due to the high variation of the data (Table 2), the settlement of larvae suggested a positive general effect of the YI extract, similar to extracts prepared in DMSO but contrasting with the results obtained for the live bacteria.

A second set of experiments was conducted to investigate if the yellow isolated bacterium produces and liberates substances that can inhibit the settlement of larvae. We used the supernatant of a centrifuged culture of the bacterium grown in liquid medium, or the filtrate of the bacterium grown in agar plates. In the first case, we did not observe a clear effect in the settlement of larvae (Fig. 3a, Table 3) after a Pearson's Chi-squared test ($X^2_{[df=2]} = 14.584$). For the second case, there was a significant effect of the filtrate over the settlement of *C. xamachana* larvae (Fig. 3b, Table 3) after a Pearson's Chi-squared test ($X^2_{[df=2]} = 8.404$). A Kruskal-Wallis test however, indicated that differences were not statistically significant (H = 2.997 for settlement, H = 4.706 for live larvae) (Table 3).

We also evaluated qualitatively the protease activity from a filtrate of cultured *Pseudoalteromonas* sp. YI, using a general serine-protease assay. Results showed that this strain releases proteases equivalent to 5-12 times the activity of 50 ng of trypsin over the same FITC-labeled casein substrate (Table 4).

Table 3 Chi-square and Kruskal-Wallis tests for released substances in larval settlement experiments

Condition of larvae	Centrifuged (df = 2)	Filtered (df =2)
Settled/Not settled	P = 0.354 [(1)]	P = 0.015 [(1)]
		P = 0.983 [(2)]
Alive/Death	P = 1 [(1)]	P = 0.007 [(1)]
		P = 0.994 [(2)]
Total number of larvae	27	136
Contingency Table	2 (S/NS, or A/D)	2 (S/NS, or A/D)
	X 3 (C, S50, S100)*	X 3 (C, F50, F100)*

df = degrees of freedom; P = p-value. Statistically significant P-values in bold.
[(1)] P-value after Pearson's Chi-square test.
[(2)] P-value after Kruskal-Wallis test.
*For contingency tables, the condition of larvae indicated as: S = settled; NS = not settled; A = alive; D = death. The experimental treatments, as: C = control; S50, S100 = 50 or 100 µL of supernatant; F50, F100 = 50 or 100 µL of filtrate.

Table 4 Qualitative evaluation of caseinolytic activity from cultured *Pseudoalteromonas* sp. Yellow Isolate. Values represent raw fluorescence units (RFU) and fold-increase of protease activity equivalent to 50 ng of trypsin acting on FITC-labeled casein substrate for samples with 25 µg mL^{-1} of soluble bacterial protein[a].

Protease Assay	50 ng trypsin	Sample 1	Sample 2	Sample 3	Sample 4
RFU	870	5081	8142	10950	7080
Fold-increase	1	5.8	9.3	12.6	8.1

[a] Protease Fluorescent Detection Kit (Sigma) using FITC-labeled casein substrate.

Discussion

We isolated a bacterial strain from the surface mucus layer of the model cnidarian *Cassiopea xamachana* identified as belonging to the genus *Pseudoalteromonas*. The isolate is pigmented and presents an apparent antibacterial activity. Several species of *Pseudoalteromonas* show dominance in biofilms; this has been attributed to their ability to rapidly form microcolonies and produce extracellular compounds with antibacterial activity (Whalen et al. 2015). We had previously observed that the surface mucus layer of *C. xamachana* was efficient at fighting tester strain *Aurantimonas* sp. even when bleached (Rivera-Ortega and Thomé 2018). The *Pseudoalteromonas* sp. yellow strain we isolated from *C. xamachana*, exhibited antibacterial activity against *Serratia marcescens* and *Aurantimonas* sp. *S. marcescens* is a long-recognized human pathogen (Hejazi and Falkiner 1997) with the ability to infect corals and cause a white pox disease (Patterson et al. 2002), giving great value to our *Pseudoalteromonas* isolated strain. The activity of this isolated strain against *Aurantimonas* might be also important. The orange to golden-pigmented species *Aurantimonas corallicida* (Denner et al. 2003) was isolated from a diseased *Dichocoenia stokesii* coral and associated with the white-plague type II disease. *Aurantimonas* was then proposed as a novel bacterial genus (Denner et al. 2003). This disease has been documented to affect massive scleractinian corals, thus other *Aurantimonas* strains like ATCC-BBA-667 may be potential coral pathogens that the *Pseudoalteromonas* sp. yellow isolate we identified and isolated seems able to outcompete. However, the pathogenicity of this tester strain to corals and other cnidarians needs to be assessed.

In general, results with different settlement assays presented high variation of the data. This can be attributed to the lack of synchronized timing in the collection of the larvae, being in general one to 3 days old. However, general trends indicated an effect of the *Pseudoalteromonas* sp. YI on larval settlement, in particular an inhibitory effect. These results are significant since other *Pseudoalteromonas* species are known for their ability to induce larval settlement in corals (Negri et al. 2001; Holmström et al. 2002; Tebben et al. 2011; Tran and Hadfield 2011). However, several *Pseudoalteromonas* species have also been found to express antifouling activity, in particular pigmented ones, aiding their hosts in avoiding the colonization of their surfaces (Holmström et al. 2002). Since we isolated this yellow strain from the mucus layer of medusas, and not from the substratum, our results are consistent with an activity that would stop larvae from settling on adult medusas. Such activity would be of benefit to the medusas, as is the antibacterial activity of this member in their microbiome. A comparison of our isolate to strains reported as inducing the settlement of coral larvae, *Pseudoalteromonas* sp. strains PS5, A3, and J010 (Negri et al. 2001; Tebben et al. 2011; Tran and Hadfield 2011; Sneed et al. 2014) resulted in identities of 99% for strains

PS5 and A3, and 98% for strain J010. These identities are lower than the identity assigned for the yellow isolate to *P. piscicida*, with 99,66 % through BLASTn. It has been reported that *P. piscicida* can inhibit the settlement of larvae from two ubiquitous fouling invertebrates, the barnacle *Balanus amphitrite*, and the tube worm *Hydroydes elegans* (Holmström et al. 2002), which may suggest a more specific inhibition of the yellow isolate for the settlement of larvae from its own host. Also, the yellow *Pseudoalteromonas* strain we isolated is 79% similar to a yellow-pigmented flavobacterium isolated from the SML of *Exaiptasia pallida* (*diaphana*) that was identified as a novel species, *Tenacibaculum aiptasiae* from a diseased anemone kept in captivity (Wang et al. 2008), clearly a different strain. Although our results suggest the *Pseudoalteromonas* sp. we isolated may affect the settlement of larvae (see Figs. 1 and 3), this effect can also be indirect, by killing bacterial strains that are needed by the larvae as settlement cues.

Proteases have been associated with the disease capabilities of human pathogenic bacteria that can substantially damage proteins of host cells and other bacteria, increasing their virulence (Backert et al. 2018). Such proteases can be secreted in soluble forms or delivered in outer-membrane vesicles. The YI indicated a 99.66% identity to *P. piscicida* that produces cysteine and serine proteases, as well as outer membrane vesicles (Richards et al. 2017), giving this species a repertoire for killing vibrios and other pathogenic bacteria. We evaluated the potential of the *Pseudoalteromonas* sp. YI to produce extracellular vesicles using a filtrate of the cultured bacteria over the settlement of *C. xamachana* larvae, finding an inhibitory effect. Although outer membrane vesicles facilitate the release of insoluble substances, they can also contain soluble substances in complex (Kulp and Kuehn 2010; Chen et al. 2020), in agreement with our results that showed an effect for a filtrate of the cultured strain but not for the centrifuged supernatant. Bacterial proteases may activate some metabolic pathways in their cnidarian hosts. For example, melanin is a general immune response in corals that helps protect them against potential pathogens; the synthesis of melanin follows the activation of the enzyme prophenoloxidase (Palmer et al. 2008). When this enzyme is secreted to the outer membrane space and locates to the surface mucus layer (Rivera-Ortega and Thomé 2018), bacterial proteases could activate it indirectly, helping the host to amplify its immunological response. However, proteases may not be the only substances delivered in outer vesicles and may also contain other inhibitors responsible for the results we obtained.

Conclusions

The *Pseudoalteromonas* strain we isolated from the surface mucus layer of the jellyfish *Cassiopea xamachana*, specifically inhibited the growth of a presumed coral pathogen and reduced the settlement of medusa larvae that we used as proxies for invertebrate larvae settlement. An organic extract prepared from a filtrate of the cultured isolate produced a 40% inhibition of the larvae as well as positive protease activity, suggesting this bacterial strain produces extracellular vesicles with antifouling compounds. These attributes must have a positive impact on the animal host, helping to fight potential pathogens and avoiding the settlement of larvae, probably foreign and own, on adult medusas. Such functions also help maintain the integrity of the surface mucus layer by halting the access of opportunistic bacteria to internal tissues that may develop into a diseased phenotype. Studies with purified bioactive compounds can clarify if this is a novel strain that may harbor novel compounds.

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Conflicts of interest The authors declare that they have no conflict of interest.

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