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Characterizing the Interactions Among a Dinoflagellate, Flagellate and Bacteria in the Phycosphere of *Alexandrium tamarense* (Dinophyta)

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A small flagellate alga was isolated from the phycosphere of a toxic red tide dinoflagellate *Alexandrium tamarense*. Phylogenetic analysis and ultrastructural observations demonstrated that the small flagellate alga is a species belong to Ochrophyte *Ochromonas* sp. The process of ingesting bacteria by *Ochromonas* sp. was recorded by a time lapse capture under a light microscope. Through the use of different assemblages in the co-culture experiment, the species interactions in this phycosphere microenvironment were analyzed. We demonstrated that the growth of *Ochromonas* sp. was supported by bacteria. Three strains of bacteria ingested by *Ochromonas* sp. were isolated and identified to belong to α-, δ- and γ-Proteobacteria. The growth of *A. tamarense* was suppressed when co-cultured with bacteria. In contrast, *Ochromonas* sp. triggered the growth of *A. tamarense* by inhibiting the growth of algicidal bacteria. This result firstly demonstrated a positive effect of a flagellate on a dinoflagellate in the phycosphere of *A. tamarense*. Combined with other negative effects between dinoflagellates and bacteria or bacteria and flagellates, this study showed a series of clear interactions among dinoflagellate, bacterium, and flagellate in the dinoflagellate microenvironment.

Keywords: ***Alexandrium tamarense*, *Ochromonas* sp., bacteria, phycosphere, species interactions**

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INTRODUCTION

Quantitative studies about the interactions among species in microecosystems are critical because these interactions control the structure and function of the ecosystem as a whole (Paine, 1966; Wootton and Emmerson, 2005). Algal-bacterial interactions are a typical model in microecosystem studies (Doucette et al., 1998; Amaro et al., 2005), in part because bacteria transform energy and regenerate inorganic nutrient in marine ecosystems (Azam et al., 1983; Su et al., 2011). *Alexandrium tamarense* is a model dinoflagellate alga that is globally distributed and known for producing harmful algal blooms (HABs) that cause paralytic shellfish poisoning (Anderson et al., 1996). *A. tamarense* growth is inhibited by certain marine bacteria, including *Brevibacterium* (Bai et al., 2011), *Thalassobius*, *Alteromonoas*, *Rhodobacteracea* (Wang et al., 2010), *Pseudoalteromonas* (Su et al., 2007b; Wang et al., 2012), *Vibrio* and *Halomonas* (Su et al., 2011). The mechanism is allelopathy, a natural phenomenon whereby a chemical released by one organism serve as a signal affecting the interaction among other organisms (Zhou et al., 2007). The allelopathy of algicidal

bacteria could be an effective way to manage HABs (Manage et al., 2001; Li et al., 2014). The allelochemicals released by *A. tamarense* tend to negatively affect competing algae (Tillmann et al., 2008; Tillmann and Hansen, 2009), though it is possible that they may positive effects on other organisms.

Aside from bacteria, dinoflagellates are also known to associate with flagellates, which sometimes consume bacteria in marine and freshwater habitats (Fenchel, 1982; Sieburth and Davis, 1982). *Ochromonas* are marine, brackish, and freshwater flagellates that contain one or two chloroplasts (Doddema and Van Der Veer, 1983; Andersson et al., 1985). However, the photosynthetic apparatus of *Ochromonas* is less efficient than that of other algae (Myers and Graham, 1956). Ultrastructural research revealed that *Ochromonas* possess highly developed phagotrophic organs, which enables efficient phagotrophy of the bacteria (Bouck, 1971; Aaronson, 1974). These characteristics suggest that *Ochromonas* plays an important role in moderating bacterial abundance through direct ingestion (Pringsheim, 1952). Additionally, some *Ochromonas* excrete antibiotic compounds (Hanse, 1973; Blom and Pernthaler, 2010).

The "phycosphere" is an environmental region extending outward from an algal cell where bacterial growth is stimulated by algal extracellular products (Bell and Mitchell, 1972). Bacteria in the phycosphere may be free-living (Blackburn et al., 1998), attached to the algal surface (Vaqué et al., 1990). The interactions, or "microbial loop" (Azam, 1998), between the algae and bacteria is intimate (Wang et al., 2010). The high level of organic compounds released by microalgae stimulate nearby bacteria, which transform these organic compounds into inorganic nutrients (Azam and Ammerman, 1984). The biomass of bacteria increase dramatically during the decline of the algal bloom, suggesting that lytic bacteria play an important role in the control and elimination of the algal bloom (Mayali and Azam, 2004; Zheng et al., 2005). Bacteria in the phycosphere can inhibit the growth of algae or even lyse algal cells in a short time (Lee et al., 2000; Mayali and Azam, 2004; Su et al., 2007a).

Because of the complex relationships between these organisms, little is known about the interactions between different algae, bacteria, and other members of the phycosphere. To characterize and quantify these interactions, we set up a time lapse microscopy recording system and used culturing techniques to check for the presence (and measure the performance) of other algal and bacteria species within the *A. tamarense* phycosphere. We found evidence for complex interactions between *A. tamarense*, *Ochromonas*, and several bacteria.

MATERIALS AND METHODS

Algal Cultures

A. tamarense (strain no.ATGD98-006) was obtained from the Algal Culture Collection, Institute of Hydrobiology, Jinan University (Guangzhou China). The alga (containing *A. tamarense* and its phycosphere microorganisms) was cultured in f/2 medium (75 mg $NaNO_3$, 5 mg $NaH_2PO_4.H_2O$, 4.36 mg Na_2EDTA, 3.15 mg $FeCl_3.6H_2O$, 0.01 mg $CuSO_4.5H_2O$, 0.022 mg $ZnSO_4.7H_2O$, 0.01 mg $CoCl_2.6H_2O$, 0.18 mg $MnCl_2.4H_2O$, 0.006 mg $Na_2MoO_4.2H_2O$, 0.1 mg thiamine HCl, 0.5 μg biotin, and 0.5 μg vitamin B_{12}, in 1 L filtered seawater, Guillard, 1975) at 20±1 °C under a 12:12 (light/dark) cycle with an illumination of 50 $\mu E/m^2$ s.

Isolation of *A. tamarense*, Flagellates and Bacteria

At mid-exponential phase, 100 mL of the algal culture was filtered through a 5-μm Isopore membrane filter. The algal cells of *A. tamarense* on the filter were immediately suspended in 50 mL of sterile f/2 medium and then purified according to a procedure for removing bacteria (Su et al., 2007a).

Flagellates in the supernatant were cultured in f/2 medium using the same conditions as mentioned above. The nutrition of mixotroph flagellate cells was maintained with bacteria in f/2 medium. Axenic cultures of the flagellates were produced by adding a mixture of antibiotics (chloramphenicol, streptomycin, and gentamycin; ratio 1: 1: 0.5) (Corno and Jürgens, 2006), followed by culture for 5 days.

To obtain bacteria cultures, 10 mL of mid-exponential phase algal cultures (containing *A. tamarense* and its phycosphere microorganisms) were filtered through a 1-μm Isopore membrane filter. The supernatant was added into Erlenmeyer flasks with 25 mL of 2216E medium (peptone, 5 g; yeast extraction, 1 g; ferric phosphorous acid, 0.1 g; agar, 10 g; pH 7.6–7.8, fixed capacity to 1 L using natural sea water), followed by incubation for 24 h at 28°C with shaking at 120 rpm.

Analysis of the Symbiotic Relationship Between Algae and Bacteria

To analyze interactions in the phycosphere, we recombined *A. tamarense*, flagellates and bacteria in a series of co-culture treatments (**Table 1**). In the co-culture experiment, the growth of each species was monitored. The initial density of *A. tamarense*, flagellates and bacteria were approximately 1.0×10^4 cell/mL, 5.0×10^4 cell/mL and 5.0×10^6 cell/mL, respectively. To determine whether *Ochromonas* can ingest dead cells of bacteria, we added cells heat-killed at 70°C for 2 h (treatment 5). Each co-culture experiment was conducted in triplicate. Samples for algal density measurements were collected every day and fixed with Lugol's iodine and counted using microscopy with a hemocytometer. Bacterial abundance was estimated by microscopic cell counting following the protocol of Patel et al. (2007).

Results were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, Washington). Averaged results were presented as mean ±SEM taken from the number of experiments indicated. Statistical significance was evaluated using Student's *t*-test. All data were from three independent experiments.

Cells of *A. tamarense* in the exponential growth phase were assigned to 24-well plates and incubated with bacteria (1.0×10^9 cell/mL). The lysis process in cells was observed by an inverted microscope equipped with a CCD camera.

TABLE 1 | Different assemblage setups for treatments.

Treatment	*Tamarense* (mL)	Flagellate (mL)	Bacteria (mL)	f/2 (mL)	Total (mL)
1. A+ /O+ /B+	15	25	5	55	100
2. A+ /O− /B+	15	0	5	80	100
3. A+ /O− /B−	15	0	0	85	100
4. A− /O+ /B+	0	25	5	70	100
5. A− /O+ /B+	0	25	5(dead)	70	100
6. A− /O+ /B−	0	25	0	75	100
7. A+ /O+ /B−	15	25	0	60	100

"A" indicates A. tamarense; "O" indicates Ochromonas sp.; "B" indicates bacteria; "+" indicates present; "−" indicates absent.

Identification of Flagellates and Ingested Bacteria

Flagellates and attached bacteria were isolated from *A. tamarense* cultures by filtering through a 5-μm Isopore membrane filter. The genomic DNA of the flagellate was extracted using the DNeasy Plant Mini Kit (Qiagen Valencia, CA, Catalog no. 69104), according to the manufacturer's suggested protocols. 18S rRNA genes were amplified using the forward primer SSU-F (5′-ACCTGGTTGATCCTGCCAGT-3′) and the reverse primer SSU-R (5′-TCACCTACGGAAACCTTGT-3′). PCR was conducted in a 50-μL reaction mixture containing 4 μL of genomic DNA, 2 μL of each primer, 25 μL of GOTaq-Colorless Master Mix, 2× and 12 μL of ddH_2O. Cyclic sequencing reactions included an initial denaturation for 10 min at 94°C, then 32 cycles of 30 s at 94°C, 45 s at 55°C, and 90 s at 72°C, followed by a final extension step of 10 min at 72°C. The PCR product was cloned into the pGEM-T Easy (Promega) vector, and the resulting plasmid was sequenced. Isolation and 16S rRNA sequencing protocols (Oh et al., 2011) were used to identify the ingested bacteria that remained within the flagellate.

Microscopy Observation

The cultured cells were centrifuged (3000 r/min, 5 min), and the medium was removed and replaced by 0.1 M PBS (pH 7.4). Cells were transferred onto a coverslip presoaked with 5% HNO_3. The slide was then stored in a specific container and 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) was added to fix the flagellates for 24 h. The glutaraldehyde solution was replaced with 0.1 M PBS (pH 7.4). After three buffer exchanges, each lasting for 15 min, the slides were taken out and naturally dried in a clean vessel. The slides were mounted on a specimen holder and dried at 30°C in a dry chamber and then sputtered with gold-palladium in a SCD Sputter Coater before examination with a JMS6390LV scanning electron microscope (JEOL, Japan).

The collected cells were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4°C and were then embedded in 2% agarose (Reize and Melkonian, 1989) and fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) over night. The glutaraldehyde solution was replaced with 0.1 M PBS (pH 7.4). After being rinsed three times for 15 min in 0.1 M PBS (pH 7.4), cells in agarose were post-fixed in 1% OsO_4 for 2–3 h at room temperature. The cells were dehydrated through a graded series of ethanol and propylene oxide, and then embedded in an Epon812 Embedding medium (3 g of DDSA, 7 g of MNA, and 10 g of Epon812 in 0.32 mL of DMP). Ultrathin sections were made using a PowerTomo-XL ultramicrotome (RMC, U.S.A.), collected on 200-mesh copper grids, and post-stained with 1% aqueous uranylacetate and lead citrate (Reynolds, 1963) before viewing with a JEM2100HC electron microscope (JEOL, Japan).

Because the small size of marine bacteria makes micrography under a light microscopy unsuitable, the samples were stained by SYBR Green I (Molecular Probes–Invitrogen) before being photographed. Fluorescence and bright field images were taken for the same vision by using a fluorescence microscope (Olympus BX-41, Olympus, Inc.).

RESULTS

Biological Characteristic of the *Ochromonas*

Light microscopy and electron microscopy (TEM and SEM) were used to investigate the morphology and ultrastructure of the flagellates. Under light microscopy, the cells swam continuously in the anterior direction and displayed a spiral path, while the long flagellum showed a curved wave, and the posterior flagellum showed no active motion but may have acted like a rudder to change direction. The cells ingesting food were non-motile because the flagellum is an important tool in capturing of particles (see Supplementary Video 1).

As shown in **Figure 1**, the flagellate cells were spherical, naked and only covered by the plasmalemma. The fluorescence microscopy images indicate that each flagellate cell contains two chloroplasts (**Figure 1B**). The cell measured 2–3 μm, and two unequal flagella arose from the anterior face of the cell. The long anterior flagellum (approximately 3–5 times the length of the cell), which bore mastigoneme (**Figure 1D**), is directed forwards. The short smooth posterior flagellum (equal to the body length), which was not easily observed under light microscopy, emerges laterally. The TEM image shows that the flagella are approximately 250 nm in diameter and that the flagellar fibers form the usual "9+2" pattern in the cross section (**Figure 1F**). The nucleus and chloroplast are enclosed by the chloroplast endoplasmatic reticulum, which may be continuous with the nuclear envelope. The chloroplast (**Figure 1E**) is bound by a double chloroplast envelope, and inside, there are arranged lamellae composed of three adpressed thylakoids. Three to four pigment granules were observed to be located on the face of the lamellae. Over half of the cell volume is occupied by the nucleus and chloroplast.

To obtain the phylogenetic information of the flagellate, 18S rRNA sequencing was performed and the 1700-bp gene fragments were compared. According to the blast results of the 18S rRNA gene sequence (GenBank accession numbers KT877396) on NCBI, 18 strains of algae from different locations with a similar sequence to the flagellate were selected to construct the phylogenetic tree (**Figure 2**). Combined with the morphology and ultrastructure result, the flagellate in this study belonged to the *Ochromonas*.

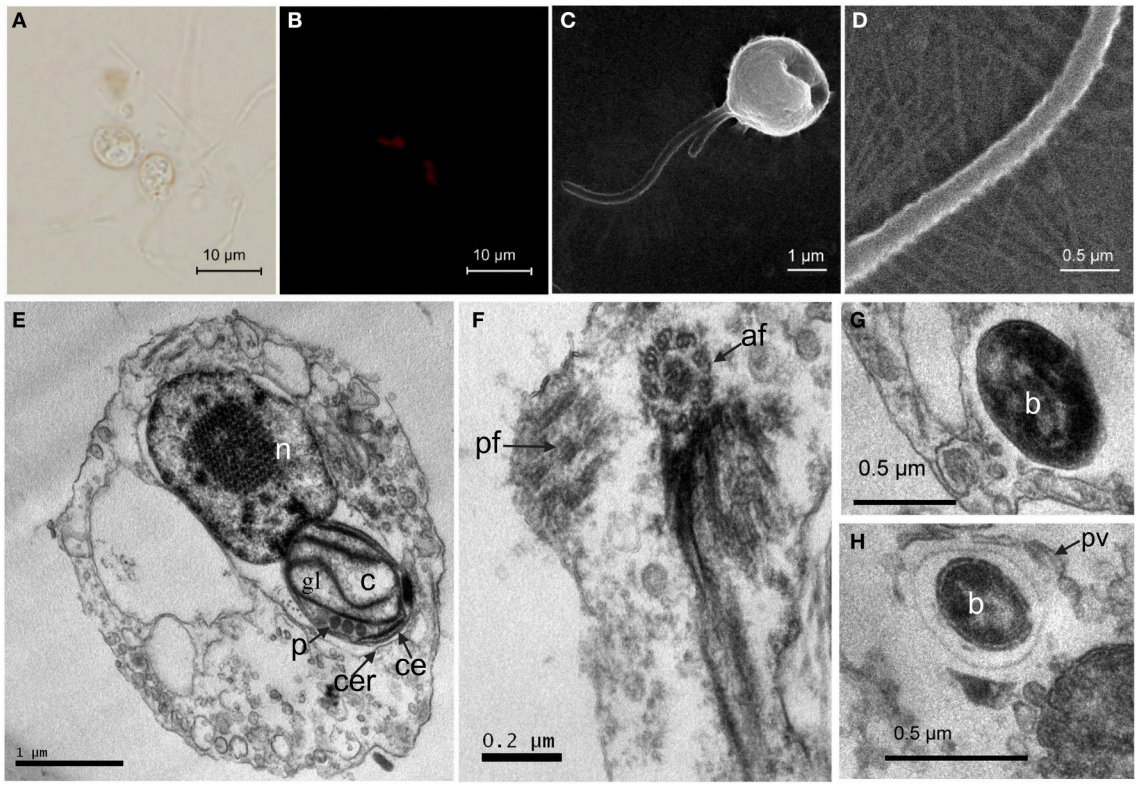

FIGURE 1 | Morphology and ultrastructure of the flagellate. (A) Bright-field image of the flagellate; **(B)** Autofluorescence of a chloroplast under the fluorescence microscopy; **(C)** SEM image showing the anterior flagellum (AF) and the posterior flagellum (PF); **(D)** High magnification view of the mastigoneme bearing AF; **(E)** Flagellate cell containing a single nucleus (n), a chloroplast (c). The chloroplast (c) is surrounded by the chloroplast endoplasmic reticulum (cer), which is connected to the nuclear envelope. The girdle lamella (gl) lies parallel to the double layers of the chloroplasts envelope (ce), and three pigment granules (p) are within the chloroplast; **(F)** Cross section of a posterior flagellum (pf), anterior flagellum (af); **(G)** Bacteria (b) within the flagellate. **(H)** Protoplasmic vesicle (pv) surrounding a bacterium (b).

Inhibitory Activity of *Ochromonas* sp. on Bacteria

The process of *Ochromonas* sp. capturing bacteria was observed (Supplementary Video 1). First, sessile flagellate cells are observed to cause the bacteria and other particle approaching them to spin by using the long anterior flagellum. Once the bacteria move into the reach of the long flagellum, they lie within a clear vesicle and are rapidly taken into the algal cell body. The *Ochromonas* sp. can capture 6–8 bacteria, and the captured bacteria moving in the cell of *Ochromonas* sp. also can be observed.

To determine whether the *Ochromonas* sp. was supported by bacteria, growth of *Ochromonas* sp. in different treatments was monitored, as is illustrated in **Figure 3**. *Ochromonas* sp. appeared to have no increase in cell density after day 1 in axenic cultures compared with cultures fed with heat-killed bacteria, which showed a slight increase in cell density of 4.93×10^4 cell/mL. In contrast, *Ochromonas* sp. co-cultured with live bacteria showed a rapid increase in growth and reached a maximum density of 2.74×10^5 cell/mL on the 15th day. In the treatment with *Ochromonas* sp. co-cultured with *A. tamarense* but no bacteria, the growth of *Ochromonas* sp. was similar to the treatment of axenic cultures. After the addition of both bacteria and *A. tamarense*, growth of *Ochromonas* sp. was suppressed at first. Then, there was a sudden growth increase on the 7th day. Moreover, it reached a maximum density of 3.26×10^5 cell/mL on the 15th day.

To investigate the impact of *Ochromonas* sp. on the bacteria, the bacterial abundance in both treatments where *Ochromonas* sp. was present and absent were estimated. The result showed bacterial growth was inhibited by *Ochromonas* sp. (**Figure 4**).

Identification of Ingested Bacteria of *Ochromonas* sp.

To obtain the phylogenetic information of the bacteria ingested by this *Ochromonas* sp., 16S rRNA sequencing and phylogenetic analysis was performed. As shown in **Figure 5**, the sequencing results indicate that isolate 1 (GenBank accession numbers KT877397) belongs to the δ-subdivisions of Proteobacteria, isolate 2 (GenBank accession numbers KT877398) exhibits 100% identity to *Roseobacter* sp. JL-351 and isolate 3 (GenBank accession numbers KT877399) shows 100% identity to *Maribacter* sp. E-8-2, which belongs to *Alteromonadaceae*.

Algicidal Effects of Bacteria on the Growth of *A. tamarense*

The effects of bacteria on the lysis of *A. tamarense* cells in the absence of *Ochromonas* sp. were observed continuously for

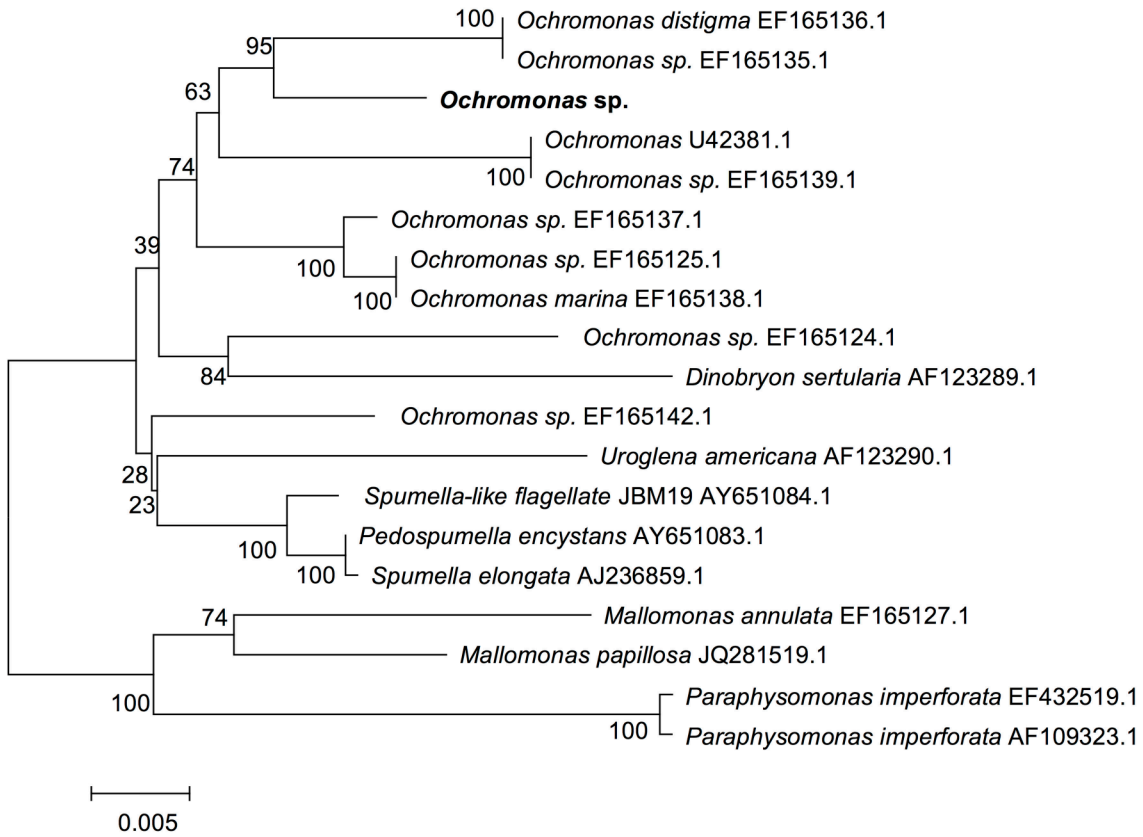

FIGURE 2 | Phylogenetic tree of the flagellate based on 18S rRNA gene sequence. Phylogenetic tree based on comparison of the 18S rRNA gene sequences indicating that this flagellate (in bold) belongs to *Ochromonas*. The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at the branching points. The scale bar indicates substitutions per nucleotide position.

24 h under light microscopy. **Figure 6** shows the morphological changes of the *A. tamarense* cells, which were incubated with bacteria at a density of 10^9 cell/mL. **Figure 6A** shows the normal cells with the integrity of the cell membrane and cell wall when the bacteria were just added. After 10 min of treatment, some cells began to break and many velum vesicles appeared on the girdle and sulcus of the cell wall and some cells escaped from their cell wall. With the exposure time increased to 30 min, the cells lysed, and the decomposed components were released from the cell (**Figure 6C**). After 24 h, most of the cells were degraded into debris and covered by a layer of bacteria.

Effects of *Ochromonas* sp. and Bacteria on the Growth of *A. tamarense*

To analyze the relationship among *A. tamarense*, *Ochromonas* sp., and bacteria, the growth of *A. tamarense* was investigated. As shown in **Figure 7**, the axenic culture of *A. tamarense* increased steadily and reached a maximum density of 3.13×10^4 cell/mL on the 19th day. In the treatment that *A. tamarense* co-cultured with *Ochromonas* sp. only, the growth of *A. tamarense* was similar to the treatment of axenic cultures. However, when co-cultured with bacteria only, a significant growth-inhibiting effect was showed on *A. tamarense,* which resulted in a lower maximum density of 1.27×10^4 cell/mL compared with the axenic culture. After adding both bacteria and *Ochromonas* sp., the growth of *A. tamarense* was similar to the axenic culture and the inhibiting effects of bacteria decreased. In these mixed cultures, *Ochromonas* sp. was observed to live close to resting *A. tamarense* cells or within the debris. Fluorescence imaging (**Figure 8B**) showed that the bacteria spread throughout the entire debris field of *A. tamarense*.

DISCUSSION

A diagnostic characteristic of the members of genus *Ochromonas* is that they are mixotrophic (Andersson et al., 1989; Zubkov et al., 2001). Heterotrophy is the major mechanism to support the growth of *Ochromonas*, and the photosynthetic apparatus is only a survival strategy during poor phototrophic conditions (Andersson et al., 1989). Some studies indicate that axenically pregrown flagellates did not significantly increase in cell number when incubated in inorganic media (Estep et al., 1986; Andersson et al., 1989). Our results showed (**Figure 3**) that the cell density of axenically cultured flagellate continuously decreased, and after 13 days, no flagellate cell was observed. By adding a certain amount of heat-killed bacteria, the number of flagellate cells slowly increased and started to decline on the 7th day (at a cell

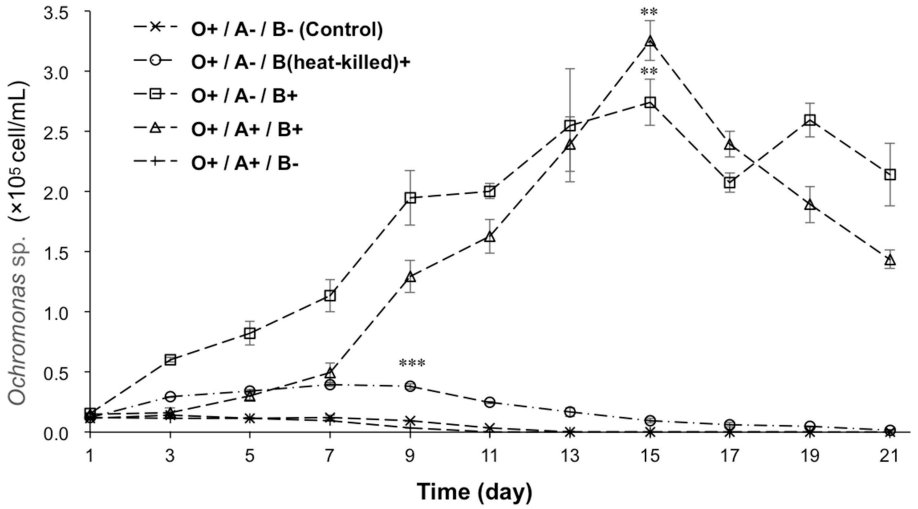

FIGURE 3 | The growth curves of *Ochromonas* sp. under the effects of bacteria and *A. tamarense*. "A" indicates *A. tamarense*; "O" indicates *Ochromonas* sp.; "B" indicates bacteria; "+" indicates present; "−" indicates absent. The asterisks **, and *** denoted statistical significance with *p*-values less than 0.01, and 0.001, respectively. All error bars indicate the SE of the three biological replicates.

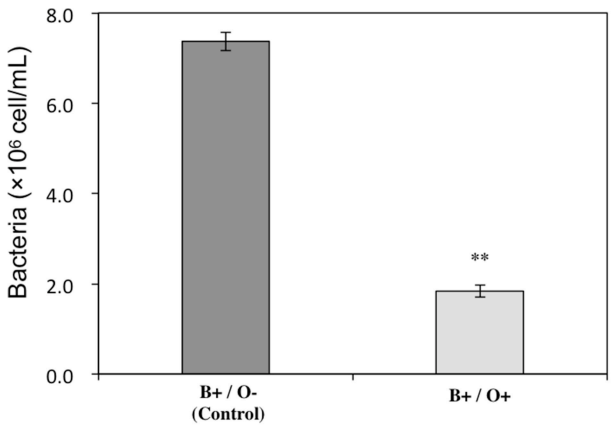

FIGURE 4 | Inhibitory effect of *Ochromonas* sp. on the bacteria cocultured with *A. tamarense*. "O" indicates *Ochromonas* sp.; "B" indicates bacteria; "+" indicates present; "−" indicates absent. The asterisks ** denoted statistical significance with *p*-values less than 0.01. All error bars indicate the SE of the three biological replicates.

density of 3.93×10^4 cell/mL). By contrast, the group that was provided live bacteria showed a rapid increase and reached a maximum density of 2.74×10^5 cell/mL on the 15th day. This is because the heat-killed bacteria provide nutrition to the flagellate in the short term, but living bacteria proliferate and steadily feed the flagellate. The results demonstrate that the autotrophic growth of the flagellate was poor.

The main nutrient strategy of this heterotrophic organism was to phagocytose small particles. Aaronson (1974) studied the phagocytic process of *Ochromonas danica* and described the vesicles and secretion as "flypaper." The ingestion of bacteria by *Ochromonas* cells is mainly attributed to vesicles secreted materials (Aaronson, 1971, 1973). As shown in the TEM images, bacteria were surrounded by vesicles with a double membrane released by the flagellate (**Figure 1H**), and then they were taken into the cell body and the vesicles were withdrawn into the cytoplasm and formed a food vacuole. Protoplasm near the anterior end of the cells tends to form several small outgrowths, as observed in **Figure 1H**, where ingestion takes place. A single flagellate cell can continuously take in numerous particles, whatever the nutrient state (Pringsheim, 1952). The whole process was first captured (Supplementary Video 1) by a CCD camera. Sessile *Ochromonas* sp. cells cause a small bacterium to approach the front of the cell by the long flagellum, and then, the bacterium suddenly stops moving and is observed to lie within a vesicle. Gavaudan (1931) describes a protoplasmic cylinder that closes over the particle and becomes a vesicle surrounding it. However, this structure was not observed in the TEM image, and the formation of the vesicle was too rapid to be seen. The vesicle carried the bacterium inside the cell body, which like a bubble, was formed at the surface of the body. This process only lasted for several seconds from the capture to the phagocytosis. The ingested bacteria kept moving within the *Ochromonas* sp. cell for a period of time, which suggests that the efficiency of digestion was low.

Grazing by small algae proved to be a major mortality factor for aquatic bacteria (Sherr and Sherr, 2002; Corno and Jürgens, 2006; Zubkov and Tarran, 2008). The *Ochromonas* diet has been described in previous studies and includes *Esherichia coli*, *Proteus mirabilis*, *Aerobacter aerogenes*, *Bacillus megaterium*, *Sarcina lutea*, and *Serratia marcescens* (Aaronson, 1974), as well as some blue-green alga (Daley et al., 1973; Li et al., 2010) and starch grains, casein, and oil droplets (Pringsheim, 1952). As a mixotrophic bacterivore, *Ochromonas* sp. showed a significant inhibiting effect on the growth of bacteria that live in the phycosphere of *A. tamarense* (**Figure 4**). To establish what

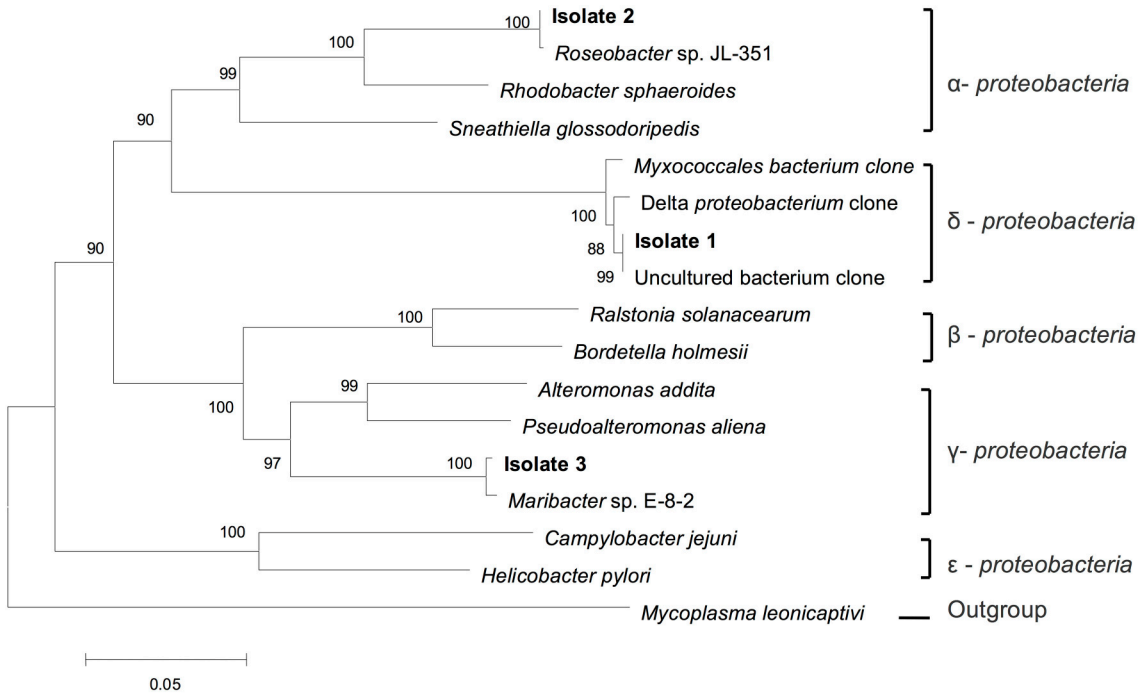

FIGURE 5 | Phylogenetic tree of the ingested bacteria. Phylogenetic tree based on a comparison of the 16S rRNA gene sequences, indicating the phylogenetic position of the strains isolated (isolation 1, 2, and 3) from flagellate culture. The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at the branching points. The scale bar indicates substitutions per nucleotide position.

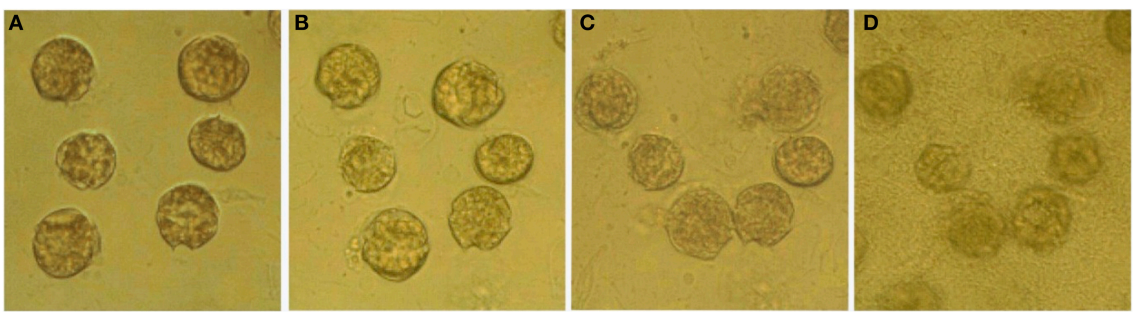

FIGURE 6 | Variation of algal structure after bacteria were added to *A. tamarense* cultures. (A–D) respectively present cells treated with bacteria for 0, 10, 30 min and 24 h.

types of bacteria are ingested, free-living bacteria were removed, and those that were ingested and remained within the cells of *Ochromonas* sp. were isolated. Phylogenetic analysis revealed that the three isolates belong to α-, δ-, and γ-Proteobacteria. Many of the α- and γ-Proteobacteria have been shown to be the phycosphere bacteria of *A. tamarense* (Mayali and Azam, 2004; Bai et al., 2011). The *Pseudomonas, alteromonas, Pseudoalteromonas*, and *Roseobacter* group, which show a high identity to our isolations, were well known to be key algicidal bacteria of *A. tamarense* and many other harmful algae (Lovejoy et al., 1998; Mayali and Azam, 2004; Amaro et al., 2005; Wang et al., 2010; Oh et al., 2011), and this finding suggests that bacteria might be useful for regulating HABs.

In natural communities, species have been found to affect each other both directly and indirectly through negative or positive interactions; thus, the study of species interactions is one of the most fundamental fields in ecology (Callaway et al., 2002). Previous reports generally focused on how negative interactions, including competition, predation and disturbance, affected the community's structure. Over the past two decades, many types of bacteria have been reported to kill algal cells by direct attack (Mayali and Azam, 2004) or produce algicidal substances (Pokrzywinski et al., 2012). These algicidal bacteria sometimes concurrently increase in abundance following the peak of some algal blooms, suggesting that negative interactions may affect algal bloom dynamics (Mayali and Azam, 2004;

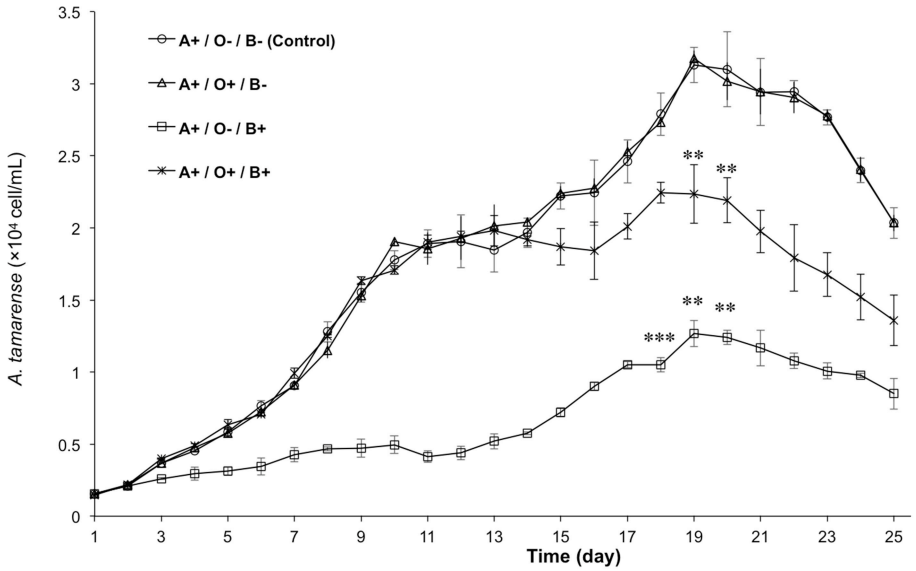

FIGURE 7 | The growth curves of *A. tamarense* under different effects of *Ochromonas* sp. and bacteria. "A" indicates *A. tamarense*; "O" indicates *Ochromonas* sp.; "B" indicates bacteria; "+" indicates present; "−" indicates absent. The asterisks **, and *** denoted statistical significance with *p*-values less than 0.01, and 0.001, respectively. All error bars indicate the SE of the three biological replicates.

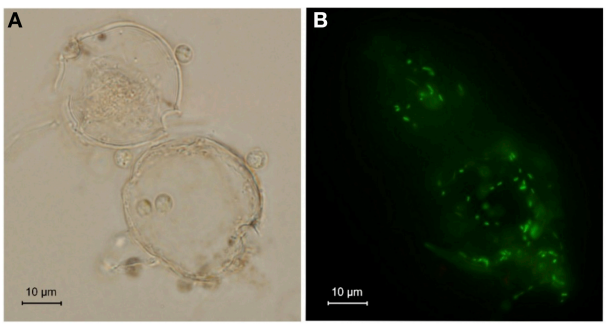

FIGURE 8 | Debris of *A. tamarense* attached with flagellate and bacteria. (A) Bright-field image. **(B)** Fluorescence image of bacteria after staining with SYBR Green I.

Pokrzywinski et al., 2012). In our experiment, the growth curves of *A. tamarense* (**Figure 7**) showed that axenic cultures of *A. tamarense* grew quickly and reached a maximum density of 3.13×10^4 cell/mL. By contrast, the growth of cells co-cultured with bacteria (at a density of 10^6 cell / mL) only reached a maximum density of 1.27×10^4 cell/mL. When treated by high-density bacteria (10^9 cell/mL), the cells of *A. tamarense* were lysed and degraded into debris within 24 h (**Figure 6**). However, some reports indicated that the algicidal activity of bacteria can be influenced by other coexisting organisms, such as predation by heterotrophic protists and competition from non-algicidal bacteria (Mayali and Doucette, 2002; Mayali and Azam, 2004). In our research, the growth of algicidal bacteria was inhibited though the predation of *Ochromonas* sp. This result revealed that there are two types of negative interactions in the phycosphere of *A. tamarense*: growth inhibition by algicidal bacteria and predation on bacteria by *Ochromonas* sp.

After adding *Ochromonas* sp. to the co-culture system, the inhibition of algal growth was relaxed (**Figure 7**) and the growth of *A. tamarense* was controlled at a maximum density of 2.24×10^4 cell/mL. Combined with phylogenetic analysis results, *Ochromonas* sp. in this study was shown to facilitate the growth of *A. tamarense* by ingesting algicidal bacteria in the phycosphere of *A. tamarense*. During our observation, cells of *Ochromonas* sp. tended to gather around the cell that was attacked by bacteria or even the debris of *A. tamarense* (**Figure 8**). Bacteria were attracted to the extracellular products or the *A. tamarense* cell itself and gathered with a high density. Quickly swimming *Ochromonas* cells do not seem to ingest food, because the flagellum plays an important role in the capture of particles and, if used in swimming, it would be inefficient for this purpose (Pringsheim, 1946, 1952). Thus, *A. tamarense* provides suitable habitat for *Ochromonas* sp. In the early period, the growth of *A. tamarense* was rapid, and a large amount of nutriments consumed by *A. tamarense* cells may disturbed bacterial growth, which indirectly affected the growth of *Ochromonas* sp. With the reduction of nutrition and accumulation of secondary products, the cell vitality of *A. tamarense* is likely weaker and more easily attacked by bacteria, while the growth of *Ochromonas* sp. also accelerated. This finding may explain the reason for the slow growth of *Ochromonas* sp. (**Figure 3**) at the early period of co-culture with both bacteria and *A. tamarense*, and the rapid growth from the 7th to the 15th days, leading to a maximum density of 3.26×10^5 cell/mL. The fact that both parties benefit suggested a mutualistic interaction in the phycosphere of *A. tamarense.* In the treatment where bacteria were absent, *A. tamarense*

and *Ochromonas* sp. both had no effect to the each other's growth. That suggested there was no direct interaction between *A. tamarense* and *Ochromonas* sp. Considering that there are negative interactions of bacteria on *A. tamarense* and negative interactions of *Ochromonas* sp. on bacteria, a positive interaction of *Ochromonas* sp. on *A. tamarense* could be recognized as emergent effect. That is to say, the growth of *A. tamarense* was controlled by the bacteria and the bacteria were controlled by *Ochromonas* sp. In general, the results of species interactions are the final outcome of a balance between negative and positive interactions, and consideration of the role played by species interactions in communities could contribute to maintaining community diversity and stability (Mulder et al., 2001; Cardinale et al., 2002; Butterfield, 2009; Cavieres and Badano, 2009).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmars.2015.00100

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