Calcium is required for ixotrophy of *Aureispira* sp. CCB-QB1

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**INTRODUCTION**

Bacterial predation plays an important role in soil and aquatic microbial ecology. Several different types of bacterial predator–prey interactions have been reported in these habitats. Bacteria such as *Bdellovibrio* (Burnham *et al.*, 1968; Sackett, 2009; Thomasow & Cotter, 1992) and *Daptobacter* (Guerrero *et al.*, 1986), which are obligate predators, penetrate the cell wall of the bacterial prey and grow within the periplasm or cytoplasm. Not all microbes kill their prey by penetrating the cell. Microbes such as *Cytophaga* sp. (Imai *et al.*, 1993; Rashid & Bird, 2001), *Myxobacteria* (Burnham *et al.*, 1981), *Ensifer* (Casida, 1982), *Herpetosiphon* (Quinn & Skerman, 1980), *Vampirovibius* (Guerrero *et al.*, 1986) and *Vampirovibius* (Esteve & Gaju, 1999) produce antagonistic chemicals or enzymes to kill prey. In the case of these facultative predators, the attacking cell does not penetrate its prey but remains outside the prey’s cell wall.

Ixotrophy is a process that enables certain microbes to prey on other cells. The ability of cells to aggregate or adhere is thought to be a significant initial step in ixotrophy. The gliding, multicellular filamentous bacterium *Aureispira* sp. CCB-QB1 belongs to the family *Saprospiraceae* and preys on bacteria such as *Vibrio* sp. in seawater. Adhesion and cell aggregation were coincident with preying and were hypothesized to play an important role in the ixotrophy in this bacterium. To test this hypothesis, experiments to elucidate the mechanisms of aggregation or adhesion in this bacterium were performed. The ability of *Aureispira* QB1 to adhere and aggregate to prey bacterium, *Vibrio* sp., required divalent cations, especially calcium ions. In the presence of calcium, *Aureispira* QB1 cells captured 99% of *Vibrio* sp. cells after 60 min of incubation. Toluidine blue O, which binds acidic polysaccharides, bound to *Aureispira* QB1 and inhibited adhesion of *Aureispira* QB1. These results suggest that acidic polysaccharides are needed for aggregation or adhesion of *Aureispira* and that calcium ions play a significant role in these phenomena.

Predation requires a cast of cellular characters, including pili, flagella and polysaccharides, whose functions in attachment and adhesion have been well documented. Attachment appears to be a critical first step in the predation process for obligate and facultative predators. Numerous studies have shown that bacterial infection on host tissues of animals or plants requires the participation of surface components such as pyelonephritis-associated pili and Afa/Dr adhesins. Both of these components are needed for enteropathogenic *Escherichia coli* to adhere to mammalian cells (Hultgren *et al.*, 1991; Pizarro-Cerdá & Cossart, 2006). Pili of *Pseudomonas syringae* facilitate attachment to the plant cells (Romantschuk & Bamford, 1986). Pili also enable cells to attach to one another to form aggregates. A dramatic example of this occurs during the development of a biofilm, which is perhaps the most sophisticated form of communal aggregation (O’Toole & Kolter, 1998).

Polysaccharides also play critical roles in adhesion, contact with extracellular surfaces, cell aggregation and formation of clumps. For example, the polysaccharide capsule (slime layer) of *Flavobacterium columnare* plays an important role in the adhesion to the gill tissue that causes columnaris disease in marine fish (Decostere *et al.*, 1999). *Azospirillum*, the free-living *N*2-fixing rhizobacterium, possesses the capacity to aggregate and flocculate. *Azospirillum* produces an exopolysaccharide (EPS) consisting of β-1,3- and β-1,4-linked glucans (Wood & Fulcher, 1978). A mutant that does not produce

**Abbreviations:** ASW, artificial seawater; CNH, CaCl2 in NH; CV, crystal violet; EPS, exopolysaccharide; NH, NaCl in HEPES buffer; SH, sucrose in HEPES buffer; TBO, toluidine blue O; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Vibrio* sp. CCB-QB3 is KT313589.

One figure is available with the online Supplementary Material.

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the EPS loses the ability to form flocs, suggesting that an EPS is critical for cell aggregation of Azospirillum (Michiels et al., 1991).

Saprospira species, gliding bacteria of the phylum Bacteroidetes, are known to prey on bacteria in aquatic environments. The marine microbe Saprospira grandis produces a sticky substance that is used to capture flagellated bacterial prey such as Vibrio. This process of capturing bacterial prey is known as ‘ixotrophy’ (Levin, 1997). Some Saprospira spp. isolated from fresh waters were able to kill aquatic bacteria; gliding bacteria such as Vitreoscilla sp. or members of the myxobacteria were particularly vulnerable (Sangkhobol & Skerman, 1981). Other species of the family Saprospiraceae have been shown to actively prey upon cyanobacteria such as Microcystis aeruginosa (Gumbo et al., 2008) and eukaryotes, such as the diatom Chaetoceros ceratoporum (Sakata, 1990).

The mechanism of ixotrophy in Saprospira spp. is poorly understood. Cells of Saprospira SS9-5, which can lyse Ch. ceratoporum, form mixed-species aggregates when both bacterial and diatom cells are present in seawater medium (Furusawa et al., 2003). EPS was visible in the aggregates by transmission electron microscopy (TEM), implicating Saprospira EPS in diatom cell capture, aggregate formation and ixotrophy.

In order to achieve a better understanding of the mechanism of ixotrophy, conditions that support ixotrophy in Aureispira QB1, a member of the family Saprospiraceae (Furusawa et al., 2015), were investigated. Aureispira QB1 aggregated quickly in artificial seawater (ASW) and was able to prey on Vibrio sp. in low-nutrient agar medium. Assays revealed requirements for divalent cations, especially calcium ions, in aggregation formation, agglutination and ixotrophy of Aureispira QB1. The results show that Aureispira is a useful model for the study of ixotrophy and aggregation in Saprospiraceae.

METHODS

Bacterial isolation and culture conditions. Aureispira sp. CCB-QB1 (to be called Aureispira QB1) was isolated from Queens Bay, Penang, Malaysia (Furusawa et al., 2015). During the enrichment and purification process, white coloured swarming colonies, reminiscent of Vibrio sp., were observed on the low nutrient artificial seawater medium (L-ASWM) (Furusawa et al., 2015) agar plates (0.05 % tryptone, 2.4 % artificial seawater mix, 1.5 % agar, 10 mM HEPES, final pH 7.6) alongside the swarms of Aureispira. The Vibrio sp. was purified from a single colony after sequential transfers to fresh medium as described for the Aureispira QB1 enrichment. Finally, growth of the Vibrio sp. was confirmed on thiosulphate-citrate-bile salts-sucrose (TCBS) agar (Kanto Chemical). Chromosomal DNA from mid exponential phase Vibrio sp. cells was prepared using the GNOME DNA kit (MP Biomedical). Phylogenetic analysis based on 16S rRNA gene sequencing was described previously (Furusawa et al., 2015).

Aureispira QB1 and Vibrio sp. were maintained on high-nutrient artificial seawater agar medium (H-ASWM) (0.5 % tryptone, 2.4 % artificial seawater mix, 1.5 % agar, 10 mM HEPES, pH 7.6) (Furusawa et al., 2015). Aureispira QB1 cells were grown to a density of $1 \times 10^8$ cells ml$^{-1}$ (OD$_{600}$= 1) in H-ASWM at 30 °C overnight. Vibrio sp. or Escherichia coli TOP10 (Invitrogen) were grown to a cell density of about $1.8 \times 10^8$ cells ml$^{-1}$ in H-ASWM overnight at 30 °C or 37 °C, respectively.

Swarming assay and single cell movement. Motility of Aureispira QB1 was quantified using an agar plate swarming assay and by time-lapse microscopy (Fremgen et al., 2010). An overnight culture of Aureispira QB1 cells was harvested by centrifugation; the cell pellet was suspended in 0.1 volume of L-ASWM to yield $1 \times 10^8$ cells ml$^{-1}$. To initiate the swarm assay, 5 μl of this suspension was spotted on 1.5 % agar L- and H-ASWM plates and incubated at 30 °C for 96 h. The diameter of each swarm was measured and the change in area (mm$^2$) was determined between $t=0$ and $t=96$ h. In addition, L-ASWM plates were supplemented with 1 mM, 2.5 mM and 5 mM EGTA to determine the role of calcium. Swarm morphology was observed using an Olympus SZX16 stereomicroscope; images were taken with an Olympus DP72 digital camera and analysed with DP2-BSW software.

Time-lapse microscopy was used to quantify single cell movement as described (Fremgen et al., 2010) with the following modifications. Fresh cells from H-ASWM plates were suspended in L-ASWM to OD$_{600}$=1 and diluted 1: 50 in L-ASWM. The cell suspension (20 μl) was placed on an L-ASWM agar plate and incubated at 30 °C for 20 min. Cells were photographed at 10 s intervals for 10 min. Motile cells (n=68) were tracked, using Metamorph 7.1 software (Molecular Devices), which was used to calculate the average speed (μm min$^{-1}$).

Predation assay on bacterial prey of Aureispira sp. CCB-QB1. Predation of Aureispira QB1 was assayed using the Queens Bay Vibrio isolate and E. coli TOP10 (Invitrogen) as bacterial targets. Aliquots of Vibrio sp. and E. coli TOP10 cell suspensions (100 μl), washed with ASW, were spread on L-ASWM agar plates and incubated at 25 °C for 1 h to allow cells to settle onto the agar surface. Aureispira QB1 cells (20 μl of $1 \times 10^8$ cells ml$^{-1}$) in L-ASWM were spotted on the bacterial lawn of Vibrio sp. or E. coli TOP10 cells. These plates were incubated at 30 °C for 144 h and plaque formation was monitored visually and microscopically.

Cell aggregation assays. Aureispira QB1 cells grown in H-ASWM were harvested by centrifugation and the cell pellet was suspended to $1 \times 10^8$ cells ml$^{-1}$ in 2.4 % ASW, or in NH (319 mM NaCl in 10 mM HEPES pH 7.6) supplemented with either 67 mM KCl, 24 mM MgSO$_4$, 12 mM MgCl$_2$, 7 mM CaCl$_2$ and no addition. These concentrations were calculated based on composition of 2.4 % of artificial seawater. SH (659 mM sucrose in 10 mM HEPES buffer at pH 7.6) was used as a control. Each Aureispira QB1 cell suspension was transferred to a 12-well plate (BD Biosciences) and immediately examined under the microscope. Fifty aggregates were chosen randomly and aggregate sizes were analysed using ImageJ software (NIH).

Aggregate precipitation was monitored spectrophotometrically to examine the kinetics of aggregation. Aureispira QB1 cells were suspended to $1 \times 10^8$ cells ml$^{-1}$ in ASW or in NH supplemented with either 7 mM MgCl$_2$, 7 mM CaCl$_2$, 7 mM CaCl$_2$ and 7 mM EGTA or no addition (negative control). The cell suspensions were incubated without shaking at 30 °C, and the OD$_{560}$ was measured at 10 min intervals for 2 h by UV spectrophotometer UV-1800 (Shimadzu). Precipitation was detected as a decrease in OD$_{560}$ as the aggregates fell out of solution.

Dye binding assay with toluidine blue O. A protocol to compare relative levels of EPS (Furusawa et al., 2011) was modified as follows. Aureispira QB1 cells were suspended to a density of about $1 \times 10^8$ ml$^{-1}$ in SH and CNH (7 mM CaCl$_2$ in NH) containing 10 μg toluidine blue O (TBO) ml$^{-1}$. All samples were vortexed for 30 s and incubated at 25 °C in the dark alongside cell-free TBO-SH and TBO-CNHa control. After 30 min of incubation, cells were removed by centrifugation and 900 μl of supernatant was transferred to a cuvette. The absorbance of each sample was measured at 630 nm for TBO and was compared with the absorbance of a cell-free control. To confirm the effect of calcium ions on the TBO bound to cells in SH, calcium ions were added to the cells suspended in SH with TBO prior to reading absorbance at 630 nm.
Adhesion assay with crystal violet. Crystal violet (CV) is routinely used to quantify bacterial adhesion or biofilm production (O'Toole & Kolter, 1998). The adhesion assay was modified for this study as follows. A 1 ml aliquot of *Aureispira* QB1 cells (1 x 10^8 cells) in SH or CNH was dispensed into 12-well microtitre plate and incubated at 30 °C for 30 min. After incubation, the suspension was gently removed so as not to disturb attached cells and a well was washed twice with the corresponding buffer. CV solution (500 μl of 0.1 % CV in 10 mM HEPES, pH 7.6) was added in each well and incubated at 25 °C for 10 min. Each well was then washed three times with 10 mM HEPES buffer (pH 7.6). After removing the buffer, 1 ml of 95 % ethanol was added and gently mixed in the well to extract the bound CV. The absorbance of each sample was measured at 595 nm. The adhesion assay with *Aureispira* QB1 cells pre-treated with TBO was conducted to determine if the TBO-binding material plays a role in aggregation formation.

Effect of calcium ions on ixotrophy in *Aureispira* sp. CCB-QB1. A method for observation of ixotrophy in *S. grandis* (Lewin, 1997) was modified in this study. *Vibrio* sp. cells grown to mid-exponential phase (OD_{600}=1, roughly 1.8 x 10^6 cells ml^{-1}) in H-ASWM were harvested by centrifugation and suspended in 1 ml of SH or CNH. *Aureispira* QB1 cells (1 ml of 10^5 cells ml^{-1}) were mixed with each *Vibrio* sp. cell suspension. An aliquot of each mixture was spotted on a glass slide and subsequently incubated at 30 °C for 20 min. The glass slide was washed three times in respective buffers and was observed by using an Olympus BX51 microscope with fluorescence imaging software Cell^{−\text{F}}.

To distinguish the number of cells captured during ixotrophy, assays were used to distinguish and quantify bound versus free-living *Vibrio* cells during incubation with *Aureispira* QB1. To count free-living *Vibrio* sp. cells, aliquots of *Aureispira*-*Vibrio* mixtures were removed after 0 min, 5 min, 30 min and 60 min incubation and an aliquot of aggregate-free cells was diluted in CNH. After an initial 1 : 100 dilution (10 μl cell into 990 μl of the buffer), two sequential 10-fold serial dilutions were made and aliquots (100 μl) of each dilution were plated on TCBS agar medium, *Vibrio* selective medium, to count c.f.u. of free-living *Vibrio* sp. cells. Colonies were scored after incubation for 24 h at 30 °C. Simultaneously, c.f.u. of *Vibrio* sp. cells from an *Aureispira*-free sample were counted as a control.

Identification and sequence similarity of *alg* genes in *Aureispira* QB1 genome. In 2014, the draft genome sequence of *Aureispira* QB1 was determined and annotated (Furusawa et al., 2015). Genes homologous to *Flavobacterium psychrophilum* JIP02/86 *alg* were found using the Rapid Annotation using Subsystem Technology (RAST) server and SEED-Viewer (Aziz et al., 2008). BLASTP analysis confirmed the amino acid similarity for *Aureispira* QB1 and *F. psychrophilum* JIP02/86 *alg* gene products.

**RESULTS**

*Aureispira* QB1 exhibits robust gliding motility

It has been reported that the type strain of *S. grandis* and *Saprospira* spp. (isolated from Kagoshima Bay) are capable of gliding and predation on other organisms when grown in low nutrient conditions (Furusawa et al., 2003; Lewin, 1997;
Sakata, 1990). Assays were performed to determine if a new isolate, *Aureispira* QB1, exhibited similar behaviour.

Aliquots of *Aureispira* were placed on agar to measure swarming (gliding group motility) and individual cell movement was examined by time-lapse microscopy. Swarming of the *Aureispira* QB1 on L-ASWM plates was reproducible with the change in area $\Delta A = 185.2 \pm 14.5 \text{ mm}^2$ after 96 h. Swarming was reduced 3.6-fold on H-ASWM agar medium with the area $\Delta A = 51 \pm 0.4 \text{ mm}^2$ (Fig. 1a). Movement of single cells on L-ASWM agar plates was tracked using Metamorph (Fig. S1, available in the online Supplementary Material). The average speed for single cells was $8.0 \pm 3.6 \mu \text{mm min}^{-1}$ ($n=68$ cells).

**Aureispira QB1 preys on bacteria**

*Aureispira* QB1 predation assays were performed using *Vibrio* sp. and *E. coli* TOP10 as a test prey. After 24 h, a small plaque (approx. 50 mm$^2$), a clearing that appears as a result of predation and death of the tester strain, was observed with the *Vibrio* cells, which increased in size (approx. 960 mm$^2$) by 144 h (Fig. 1b). Inside the plaque, *Aureispira* QB1 cells were dominant and formed a meshwork that enveloped the *Vibrio* sp. cells (Fig. 1c, Inside). At the boundary area between the clear zone and the growth, *Aureispira* QB1 cells appeared to envelop the *Vibrio* cells (Fig. 1c, Boundary). The plaque formation was similar to that of *Saprospira* sp. SS98-5 against the diatom *Ch. ceratosporum* (Furusawa et al., 2003) or *Myxococcus xanthus* PCO2 against the cyanobacterium *Phormidium luridum* (Burnham et al., 1981). *Aureispira* QB1 also showed predation on commercial *E. coli* TOP10 (Fig. 1b).

**Divalent cations affect aggregate formation of *Aureispira* sp. CCB-QB1**

Previously, it was reported that *Saprospira* sp. SS98-5 could aggregate with diatom cells under low nutrient condition (Furusawa et al., 2003). Similarly, *Aureispira* QB1 cells aggregated immediately (<1 s) when cells were suspended in ASW. These aggregates disappeared when samples were washed with water but formed again when cells were resuspended in ASW. This shows that aggregation was reversible. In addition, to examine the effect of individual ions included in artificial seawater, cells were suspended in media containing each ion, such as MgSO$_4$, MgCl$_2$, CaCl$_2$ and KCl. As shown in Fig. 2, aggregate size was variable and depended upon the type of cation in the medium. Aggregates of *Aureispira* QB1 in ASW were $25.5 \pm 25.1 \mu \text{m}^2$, while aggregates in buffer containing only MgCl$_2$ and CaCl$_2$ yielded aggregates that were $27.0 \pm 33.9 \mu \text{m}^2$ and $23.0 \pm 22.6 \mu \text{m}^2$, respectively. Smaller aggregates formed in MgSO$_4$ ($10.9 \pm 9.8 \mu \text{m}^2$), NaCl ($2.5 \pm 1.5 \mu \text{m}^2$) and KCl (too small to measure) solutions. In fresh water or sucrose buffer, cells did not aggregate (see Fig. 2 for sucrose). These results indicated that ions, especially divalent ions, are necessary for aggregation formation.

The effect of divalent ions on the kinetics of aggregate formation was measured spectrophotometrically. Precipitation was monitored as the change in optical density (OD$_{600}$) over time. As shown in Fig. 3(a), cells suspended in CaCl$_2$ solution aggregated quickly at about 0.6 OD units h$^{-1}$. Cells precipitated in MgCl$_2$ solution. The rate of precipitation in 7 mM CaCl$_2$ with 7 mM EGTA was less than 0.1 OD units h$^{-1}$.

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**Fig. 2.** Influence of cations on cell aggregation of *Aureispira* QB1. Cell aggregation was observed in each buffer including cations. ASW or sucrose buffer was used as positive or negative control, respectively. Bars, 0.5 mm.
To test the effect of calcium ions on aggregate formation, cells were suspended in 1 mM, 2.5 mM, 5 mM, 7 mM and 10 mM CaCl₂ solution. Cells suspended in 7 mM and 10 mM CaCl₂ solution aggregated faster than that of other conditions.

Based on these results, calcium ions, provided as CaCl₂ (7 mM), were selected as the divalent cation in additional tests on dye binding, adhesion and isotrophy.

**Influence of calcium ions on production of extracellular substrate**

TBO is generally used in histology for cell walls of plant, mast cell or cartilage and is one of the most common stains for acid mucopolysaccharides. The TBO dye-binding assay was performed to determine if *Aureispira* QB1 produced acidic polysaccharides. As shown in Fig. 4, cells in SH bound about 80 % of the TBO while cells in CaCl₂ bound 12 % of the TBO, a decrease of over sixfold. In addition, the bound TBO decreased by about 20 % after addition of CaCl₂ to cells in SH. These results show that *Aureispira* QB1 cells bind TBO and that calcium ions competitively affect TBO binding. Acidic polysaccharides may be the TBO binding material on *Aureispira*.

**Calcium ions stimulated adherence of *Aureispira* QB1**

To determine the adhesive capabilities of this bacterium, formation of aggregates was monitored using the CV spectrophotometric analysis. As shown in Fig. 4, aggregates formed in CNH and can be seen attached to the bottom of the culture plate after CV staining. In contrast, very few of the cells in SH were found to adhere to the bottom of culture plates (Fig. 5). The absorbance (OD₅₉₅) due to CV of the sample in CNH was about 11 times higher than that of SH. This result indicates that calcium ions are essential for adherence of *Aureispira* QB1.

To determine if the TBO-binding material plays a role in adhesion, the adhesion assay was conducted with *Aureispira* QB1 cells pre-treated with TBO. The absorbance (OD₅₉₅) of the TBO-binding *Aureispira* QB1 cells (OD₅₉₅=2.81) was nine times lower than that of cells without TBO (OD₅₉₅=0.34). This result hints that adhesion of *Aureispira* QB1 may require the function of material that binds to TBO.
Aureispira QB1 cells showed activity of ixotrophy in the presence of calcium ions

As shown above, calcium ions play an important role in aggregation and adhesion of Aureispira QB1 cells and may affect the function of EPS of the cell. Based on these results, we anticipated that calcium would be obligatory for ixotrophy of Aureispira QB1. To confirm these expectations, Aureispira QB1 cells and Vibrio sp. cells were mixed together in CNH. Neither bacterial strain in SH was able to attach to the glass slide (Fig. 6a). In contrast, in the presence of calcium ions, individual Aureispira QB1 cells attached to the surface of the glass slide and Vibrio sp. cells were found in close proximity to Aureispira QB1 cells (Fig. 6a). The number of Vibrio sp. cells captured by Aureispira QB1 cells in mixed culture was measured. Fig. 6b shows the changes in the number of free-living Vibrio sp. cells in mixed culture over time. By comparing the number of free-living Vibrio sp. cells in mixed culture and that of Aureispira-free sample, we calculated that 82 % of Vibrio sp. cells were captured by Aureispira QB1 cells after 5 min of incubation (solid squares) and 99 % Vibrio sp. cells were captured by Aureispira QB1 cells after 60 min. Hence, Aureispira QB1 exhibits calcium-dependent ixotrophy.

Decreasing concentration of calcium ions negatively affects swarming motility and plaque formation

The role of calcium ions on swarming motility and plaque formation was also examined. Aureispira QB1 cells showed swarming motility on L-ASWM agar plates with 1 mM and 2.5 mM of EGTA; however, swarming of the cells was inhibited in the presence of 5 mM EGTA (Fig. 7a).

The ability of Aureispira QB1 to form a plaque on Vibrio produced a response on EGTA medium that paralleled the swarming response. Similar size plaques formed on 0, 1 and 2.5 mM EGTA, but were significantly smaller on L-ASWM agar plates with 5 mM EGTA (Fig. 7b). This suggested that swarming and ixotrophy both share a requirement for a calcium-requiring component in Aureispira QB1.

DISCUSSION

Cell attachment and/or aggregation play an important role in bacterial predation. The genus Saprospira is a model microorganism of ixotroph-type bacterial predation. However, the mechanisms of attachment or aggregation of Saprospira remain poorly understood. Although Aureispira marina, belonging to the family Saprospiraceae, has been described as the gliding and arachidonic-acid-containing bacterium (Hosoya et al., 2006), it has not been reported whether Aureispira spp. are bacterial predators. In this study, predation of Aureispira QB1 on environmentally relevant organisms was demonstrated. Aureispira QB1 showed rapid aggregation in ASW and it was found that divalent cations, especially calcium ions, were required for efficient aggregation.

Bacteria that synthesize the acidic polysaccharide, alginate, have been reported mainly in two genera, Pseudomonas and Azotobacter (Remminghorst & Rehm, 2006). Genetic analysis of alginate biosynthesis in Ps. aeruginosa has identified alg genes that are involved in synthesis of precursor substrate (algA, algC and algD), polymerization (algK), modification (algF, algG, algJ, algL and algX) and export (algE) (Remminghorst & Rehm, 2006; Riley et al., 2013). A two-component signal-transduction pathway that regulates alg operon transcription was found (Rehm & Valla, 1997).

Aureispira QB1 contains homologues of some alg genes. Table 1 shows that algC, algF and algJ are present in Aureispira QB1 and F. psychrophilum JIP02/86, a fish pathogen and algA was present only in Aureispira QB1. Amino acid sequences of algC, algF and algJ exhibited high similarity (> 50 %) to that of F. psychrophilum JIP02/86. These four genes maintained highly conserved domains in each enzyme. Duchaud et al. (2007) suggested that the F. psychrophilum alg genes might function in biofilm formation. Nichols et al. (2005) have reported that bacteria belonged to the family Flavobacteriaceae
produced EPS including uronic acid, which is a component of alginate. Taken together, these results hint that *Aureispira* QB1 may have the ability to produce a polysaccharide related to alginate. This is consistent with the finding that *Aureispira* QB1 bound TBO, a basic thiazine metachromatic dye that is one of the most common stains for acidic mucopolysaccharides, such as alginate and glycosaminoglycans. Similarly, the ability of calcium to stimulate aggregation and adhesion of *Aureispira* QB1 and *Vibrio* may be related to the fact that complexation by calcium stimulates swelling of carbohydrate gels such as alginate. Alternatively, calcium may help to neutralize the electrical forces that might otherwise cause interference between bacterial cells. The role of repulsive forces between negatively charged bacterial cells or between the cells and solid surface (i.e. a tissue culture well) has been described (Geesey et al., 2000).

Lewin demonstrated that *S. grandis* was able to catch bacterial prey in the absence of calcium ion (Lewin, 1997). In addition, Aizawa and colleagues reported that thin, membranous sheath-covered filaments in *S. grandis* were postulated to play a role in capturing prey by entanglement (Aizawa, 2005, 2013; Bourret et al., 2002). However, the results of this study suggest that calcium ions stimulated cell aggregation and adhesion related to ixotrophy of *Aureispira* QB1. *Aureispira* QB1 cells required calcium ions for capturing *Vibrio* sp. cells. Capture of prey was rapid, with over 80% of *Vibrio* consumed within 5 min. Moreover, *Aureispira* QB1 cells formed a plaque on a lawn of

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**Fig. 6.** Observation of ixotrophy of *Aureispira* QB1 towards *Vibrio* sp. requires calcium ions. (a) Samples were incubated on glass slides and were washed with the same buffer. Right: sample was incubated in SH. Left: sample was incubated in CNH. The ixotrophy was observed by phase-contrast microscopy. Bars, 20 μm. (b) The number of free-living *Vibrio* sp. cells in mixed culture was monitored over time. Colony-forming units of free-living *Vibrio* sp. cells were determined by removing aliquots of cells after 0, 5, 30 and 60 min of incubation. Filled squares or diamonds indicates samples with or without *Aureispira* QB1 cells, respectively. Values are mean ± SD from three independent biological replicates.
**Fig. 7.** Swarming and plaque formation of *Aureispira* QB1 in the presence of EGTA. Comparison of swarming area (a) and plaque formation (b) of *Aureispira* QB1 on a lawn of *Vibrio* sp. cells on L-ASWM with 0, 1, 2.5 and 5 mM EGTA. Experiments were repeated three times. Bars, 2 cm. Values of swarming areas are mean ± SD from three independent biological replicates.

**Table 1.** Similarity of amino acid sequences of *alg* gene products in *Aureispira* QB1

<table>
<thead>
<tr>
<th>Gene product</th>
<th>ID (RAST)</th>
<th>Function</th>
<th>Similarity to <em>F. psychrophilum</em> homologues (%)</th>
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<td>Mannose 6-phosphate isomerase</td>
<td>No similarity</td>
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Vibrio sp. cells plaque formation may be a kind of ‘group predation’ whereby a number of predator cells cooperate to produce hydrolytic enzymes that lyse nearby bacterial prey (Dworkin, 1996; Guerrero et al., 1986). Efforts to identify predation induction signals and the Aureispira QB1 responses are under way.

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REFERENCES


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Influence of calcium ions on bacterial predation