

Carbohydrate-degrading bacteria closely associated with *Tetraselmis indica*: influence on algal growth

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ABSTRACT: In the present study, we examined the interactions between the algal species *Tetraselmis indica* and strains of bacteria with which it is closely associated. Three bacterial strains were isolated and sequence analysis of the 16S rDNA indicated that the organisms belong to the genera *Pseudomonas*, *Acinetobacter* and *Ruegeria*. Morphologies of the bacterial strains were studied using epifluorescence microscopy and scanning electron microscopy. Reassociation experiments were conducted with axenic cultures inoculated with the 3 bacterial strains in concentrations comparable to natural conditions, and the effect of each bacterial population on the growth of *T. indica* was determined. *T. indica* exhibited differential growth with the various bacterial cultures, and in particular *Acinetobacter* sp. was observed to promote growth of the algae. These experiments revealed that microbes associated with the alga differentially influence algal growth dynamics. Bacterial presence on the cast-off cell wall products of the alga suggested the likely utilisation of algal cell wall by bacteria. The bacterial strains were tested for carbohydrate metabolism using various sugars and screened for carbohydrase activity. Bacterial strains were found to produce carbohydrases for degradation of polysaccharides generally present in the cell wall of *Tetraselmis* (glucans, galactans, galactomannans and pectins), whereas no such utilisation was observed for other wall substrates (such as cellulose, arabinoxylan, rhamnogalacturonan). *Pseudomonas* sp. and *Acinetobacter* sp. showed carbohydrase activity with glucans, galactans, galactomannans and pectin, whereas *Ruegeria* sp. showed much less carbohydrase activity and only with pectin. The carbohydrate utilisation studies using artificial substrates suggested the potential utilisation of cast-off algal cell wall products.

KEY WORDS: *Tetraselmis indica* · Aquatic ecosystems · Associated bacteria · Reassociation experiments · Carbohydrate utilisation · Algal growth

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INTRODUCTION

Phytoplankton release considerable amounts of organic components into the extracellular medium, most of which are utilised by bacteria (Williams 1981, Coveney 1982, Cole et al. 1988, Coveney & Wetzel 1989, Ducklow & Carlson 1992, Kormas 2005). Consequently, heterotrophic bacteria are

often found in close association with algae, which are able to provide a favourable habitat for these bacteria within aquatic ecosystems (Caldwell 1977, Paerl & Pinckney 1996). The possible role of bacteria in enhancing the growth of algae has been documented (Cid et al. 1992, del Giorgio et al. 1997, Moreno & Laine 2004, de Kluijver et al. 2010). Lange (1967, 1970, 1971) proposed that bacteria

may be a significant source of CO₂ for algal growth during periods of carbon limitation.

The taxa of bacteria that can be cultured from samples of phytoplankton are often different from those that can be cultured from water samples (Simidu et al. 1971, Kogure et al. 1981, Cole 1982). Specific bacteria are habitually associated with phytoplankton and are highly adapted for utilisation of extracellular algal products (Bershova et al. 1968, Kuentzel 1969, Bell & Mitchell 1972, Bell & Sakshaug 1980, Meffert & Overbeck 1985, Maurin et al. 1997, Aota & Nakajima 2001, Huang et al. 2004, Zifeng et al. 2009). Heterotrophic utilisation of algal products can be expected to be a function of both the activity of the heterotrophs and of the suitability of the excreted material (Coveney 1982). In addition to the exudation of organic components (Mague et al. 1980, Bjørnsen 1988, Myklestad et al. 1989, Obernosterer & Herndl 1995), algal components released through cell lysis (Brussaard et al. 1988) and the cast off theca (Lewin 1958, Gooday 1971, Becker et al. 1991) may be an important carbon source for bacteria.

In the present study, we examined the interactions between an algal species and the strains of bacteria with which it is closely associated under controlled conditions. The alga used for this purpose was *Tetraselmis indica* (M. Arora et al. unpubl.) collected from a salt pan in Goa, India. During the non-motile phase of this alga, new walls develop and old walls are cast off and accumulate as concentric rings surrounding the cell.

The bacterial strains isolated from *Tetraselmis indica* were grown in combination with axenic cultures of *T. indica* and the interactions between bacteria and algae were examined. In order to test the hypothesis that bacteria degrade and utilise cast-off algal cell wall products, the bacterial utilisation of carbohydrates was investigated.

MATERIALS AND METHODS

Isolation of culturable planktonic and epiphytic bacteria

Tetraselmis indica cultures were maintained in F/2 media (Guillard & Ryther 1962) without silicates at 25°C with a photon flux density of 80 μmol photons m⁻² s⁻¹ and a 16 h light:8 h dark cycle. For isolation of bacteria, 500 ml of *T. indica* culture (in sterile F/2 algal culture media) was sonicated using a sonicator (Ultrawave U300H with ultrasonic power 35 W and operating frequency 44 KHz) for 5 min and filtered

through a sterile GF/C (1.2 μm) Whatman glass fibre filter. The filtrate was again filtered through a 0.2 μm Whatman membrane filter whilst under vacuum. The 0.2 μm filters containing bacteria were then washed using 2 ml of sterile seawater and mildly sonicated (for 3 min). Serial dilutions (10⁻¹ to 10⁻⁶) were prepared using sterile seawater. A 0.1 ml aliquot of each dilution was then plated onto marine agar (Difco) and on various selective media in triplicate under aseptic conditions. As controls, 0.1 ml aliquots of 0.2 μm filtrate and F/2 media were also plated on marine agar and selective media. Marine agar was chosen as a generalised medium to grow all the culturable bacteria from algal culture. Depending on the source of isolation, any micro- or macroalgae from seawater may contain culturable coliforms, vibrios, as well as other heterotrophic bacteria such as *Shewanella*, *Alteromonas*, *Planococcus* etc. We therefore chose to use:

- (1) A-1 medium (Difco) for growth of coliforms including *Escherichia coli*,
- (2) Thiosulfate citrate bile salts sucrose agar (TCBS, Difco) for growth of *Vibrio* spp.,
- (3) *Pseudomonas* isolation agar (Difco) for growth of pseudomonads,
- (4) Sea-water agar (SWA, DSMZ, 246) (Difco) for growth of heterotrophic bacteria.

Marine agar, TCBS, A-1 and SWA plates were incubated at 25°C for 24 h and the plates for selective pseudomonad growth at 37°C for 48 h.

Identification and phylogenetic analysis of bacteria

Three characteristic bacterial morphologies were identified from all the replicates of marine agar plates, representing the diversity of bacteria associated with *Tetraselmis indica*. Single colony morphology of similar type was observed in each plate of A-1 medium, *Pseudomonas* isolation agar medium and SWA. Single colonies from each plate were streaked for pure strain isolation. Identification of bacterial strains was performed by 16S rDNA sequence analysis. Amplification of the 16S rRNA gene sequences was performed using genomic DNA as the template, extracted using the standard alkaline-lysis protocol from whole bacteria (Birnboim & Doly 1979). The 16S rRNA gene was PCR-amplified for all strains using universal prokaryotic primers: 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' (*E. coli* 27–47) and 1492R 5'-TAC GGT TAC CTT GTT ACG ACT T-3' (*E. coli* 1492–1514) (Lane 1991). The amplification reactions were carried out using a QIAGEN Fast Cycling PCR Kit and an Eppendorf Mastercycler PCR machine. Dye terminator se-

quencing, using the same primers as in the amplification step, and an Applied Biosystems 3730xl DNA Analyzer at Newcastle University were used to obtain the nucleotide sequences. 16S rDNA sequences were then compared with those available in the National Centre for Biotechnology Information (NCBI) database using the BLASTN algorithm (Altschul et al. 1990), and the percentage identity was determined. Consensus sequences were aligned using CLUSTALW2 (Hall 1999, Larkin et al. 2007). The TrN+I+G (Tamura-Nei) Model was selected using MEGA v5 (Tamura et al. 2011) and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by the maximum likelihood method. Neighbour joining and maximum parsimony trees were also inferred using MEGA. The sequences obtained were submitted to NCBI GenBank under the accession numbers JF828049, JF828050 and JF828051 for *Pseudomonas*, *Acinetobacter* and *Ruegeria* sp., respectively.

Morphological study of bacteria

Cultured cells were sampled during the late exponential growth phase. Cells were stained using SYBR Gold stain at a working dilution of $10 \mu\text{l ml}^{-1}$ (Molecular Probes, Invitrogen, 1:100 dilution in distilled water) and observed under epifluorescence microscopy. For scanning electron microscopy (SEM), cells were fixed in 2% glutaraldehyde (TAAB Laboratory Equipment), cacodylate and seawater. Cells were allowed to settle on poly-L-lysine-coated coverslips before placing the coverslips in a holder and dehydrating the cells in an ethanol series (10 min each in 25%, 50% and 75% followed by twice for 15 min in 100%). The coverslips were dried in a critical point dryer (BalTec) and subsequently mounted on stubs with a silver DAG and carbon disc (Agar Scientific). Finally, the cells were sputtered with gold (using a Polaron SEM coating unit) and observed using a Stereoscan S40 Scanning Electron Microscope (Cambridge Instruments) at the Electron Microscopy Unit of the School of Biomedical Sciences, Newcastle University.

Growth in axenic culture with different types of bacteria

Preparation of axenic and xenic cultures of phytoplankton

Tetraselmis indica cells were washed (5 times) with sterile seawater before and after mild sonication (3

min) to remove closely adhered bacterial cells. Cultures were incubated in carbenicillin (0.02 g l^{-1}) for 24 h, then were given a sterile wash. Cultures were allowed to recover for 6 d by incubating in sterile F/2 algal culturing medium (Guillard & Ryther 1962) at 20°C before being incubated in fresh sterile F/2 medium containing 0.1 g l^{-1} cefotaxime for a further 24 h at 20°C . Cultures were given 5 repeated sterile washes and transferred to antibiotic-free F/2 medium. The purity of the algal cultures was tested using 3 methods: first, the spread plate method using Zobell marine agar (Himedia); second, by microscopic investigation after fluorochrome staining the cultures using 4',6-diamidino-2-phenyl indole (DAPI) and acridine orange; and third, by flow cytometry using SYBR Gold fluorescent stain (Molecular Probes, Invitrogen) and a BD FACS Aria II flow cytometer. For flow cytometry, samples were run at a rate of $10 \mu\text{l min}^{-1}$ on a FACS Aria II flow cytometer (Becton Dickinson) equipped with a 488 nm excitation laser and a standard filter setup. To create xenic cultures of algae (microalgal cultures containing bacteria), axenic cultures (75 ml each) were inoculated directly at the start of the experiments with a mixed community of the bacteria (presumably culturable as well as non-culturable) present in the original isolate. The mixed community of marine bacteria was obtained from the sonicated culture at the end of the exponential phase by filtration through a sterile GF/C Whatman glass fibre filter (Brussaard et al. 1998). The filtrate was then used as the bacterial inoculum and was added into axenic culture for preparation of xenic cultures of *T. indica*. The mixed bacterial community therefore consisted of a group of bacterial species originally present in the culture and adapted to the specific culture conditions. The concentration of bacteria at the start of the experiments was between 0.45 and $0.6 \times 10^6 \text{ cells ml}^{-1}$. A control was prepared using the axenic culture which received 5 ml of the $0.2 \mu\text{m}$ filtrate containing no bacteria.

Estimation of bacterial and algal population by counting

For the estimation of bacterial biomass, all cultures were subjected to mild sonication and filtered through $1.2 \mu\text{m}$ GF/C filters before the optical density at 590 nm was measured. Bacterial cell counts were obtained using epifluorescence microscopy and the acridine orange direct count method (Hobbie et al. 1977). Cell counts were also

calculated by staining the cells with SYBR Gold (Molecular Probes, Invitrogen) and using flow cytometry.

To study the effect of each bacterial type on the phytoplankton population in terms of cell numbers, growth dynamics of axenic culture and xenic culture were studied under standard culture conditions. A single colony from each bacterial plate was suspended in 1 ml of sterile seawater which was serially diluted to give a range of 10^{-1} to 10^{-6} cells ml^{-1} . Each strain of bacteria was prepared at a density of 3.2×10^6 cells ml^{-1} . Then 5 ml of each bacterial inoculum was added to 75 ml of axenic culture to give a final bacterial concentration of 0.4×10^6 cells ml^{-1} , a comparable concentration to those of xenic cultures. Total cell counts were performed using microscopy and phytoplankton growth was considered as being in the stationary phase when the cell counts did not differ significantly for 3 successive days. Algal growth during exponential, stationary and senescence phases was studied using cell counts calculated with a haemocytometer. For both xenic and axenic cultures, specific growth rates (K') for Days 10 to 18 (exponential phase) of the experiment were calculated according to Levasseur et al. (1993) using the following formula and were averaged:

$$K' = \ln(N_2/N_1) / (t_2 - t_1) \quad (1)$$

where N_1 and N_2 are the cell counts at times (d) t_1 and t_2 , respectively.

A comparison of dead cell counts of axenic culture in combination with various bacteria was performed using SYTOX Green stain (Molecular Probes, Invitrogen) and a BD FACS Aria II flow cytometer. Samples were run at a rate of $10 \mu\text{l min}^{-1}$ on a FACS Aria II flow cytometer (Becton Dickinson) equipped with a 488 nm excitation laser and a standard filter setup.

Carbohydrate utilisation (mono- and disaccharide)

Biochemical assays were performed on all 3 bacterial strains isolated to test their ability to metabolise the various carbohydrates (see Table 1) resulting in the formation of an acid and the production of CO_2 . The medium used for these assays contained peptone (10 g), beef extract (1 g), NaCl (5 g) and phenol red (0.018 g). The above-mentioned components were dissolved in 500 ml distilled water and the volume made up to 1 l. This broth was sterilised in an autoclave at 120°C for 15 min. Solutions of each sugar (10%) were prepared by adding 2 g to 20 ml distilled water and autoclaving below 100°C . Then 4 ml of

phenol red broth and 1 ml of each sugar solution were dispensed into tubes containing Durham gas-collecting tubes for gas detection. A sample of each of the assigned bacteria was aseptically inoculated into each of the phenol red broth tubes, using a loop.

Following incubation, carbohydrates that had been metabolised produced an acidic waste product causing the phenol red to turn yellow, indicating a positive reaction. In certain cases, acid production was accompanied by the evolution of gas (CO_2) which was visible as a bubble in the inverted tube.

Evaluation of the biodegradability of carbohydrate substrates (polysaccharides)

Eight types of biodegradable substrates (see Table 2) were used in this study; azo-galactan, azocarob galactomannan, AZ-rhamnogalacturonan, azo-CM-cellulose, red pullulan, wheat arabinoxylan, β -glucan (Megazyme) and citrus pectin (Sigma) were added to the culturing media SWA at a concentration of 2.5 g l^{-1} . Pure colonies of each bacterium were streaked onto media in triplicate and incubated at 37°C for 48 h. Cleavage of the chromogenic substrates by bacteria produced a halo-like precipitation zone around the bacteria, indicating positive activity. Non-chromogenic substrates were stained using 1% congo red for 15 min and a similar halo-like precipitation was observed following destaining with 1 M NaCl in cases of positive activity.

RESULTS

Isolation, identification and phylogenetic analysis of bacteria

Three media, A-1, *Pseudomonas* isolation agar and SWA, demonstrated that 3 morphologically different bacterial colonies were present. Similar colonies were observed on marine agar. TCBS agar did not yield any bacterial growth, indicating the absence of vibrios in culture. Sequence analysis of 16S rDNA from duplicates indicated that the organisms isolated from *Tetraselmis indica* belonged to the *Acinetobacter*, *Ruegeria* and *Pseudomonas* genera. The *Pseudomonas* sp. showed maximum sequence similarity to *P. stutzerii* (98%), the *Acinetobacter* sp. to *A. haemolyticus* (98%) and the *Ruegeria* sp. to *R. atlantica* (97%) (Fig. 1 and see Supplement 1 at www.int-res.com/articles/suppl/b015p061_supp.pdf).

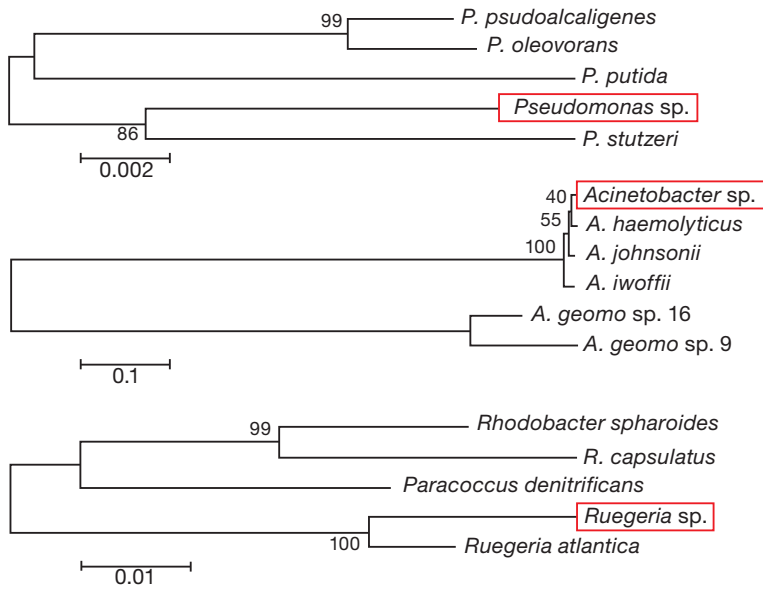


Fig. 1. *Acinetobacter* sp., *Pseudomonas* sp. and *Ruegeria* sp. Phylogeny of the 3 bacterial strains based on 16S rDNA sequences, showing their position in relation to the closest species of their respective genera. Original strains sequenced in this study are highlighted using red squares. The phylogenetic tree shown was inferred by the maximum likelihood method based on the Tamura & Nei (1993) model of DNA substitutions

Morphologies with epifluorescence microscopy and SEM

The 3 isolates were observed by epifluorescence microscopy and SEM. All morphological features observed were typical of the corresponding genera. Morphologies as observed under epifluorescence microscopy correlated well with those viewed by SEM. *Acinetobacter* sp. was seen to be present in pairs (Fig. 2A,B) as expected for members of the *Acinetobacter* genus (Carr et al. 2003), whilst *Ruegeria* sp. was observed to be spherical in shape and readily formed chains (Fig. 2C,D). *Pseudomonas* sp. demonstrated a rod-shaped morphology (Fig. 2E,F). *Pseudomonas* colonies were hard and dry with a characteristic wrinkled appearance as expected for *Pseudomonas stutzerii* (Lalucat et al. 2006). The occurrence of irregular polygon-like structures or concentric zones was also noted, as has been described earlier for *Pseudomonas stutzerii* (van Niel & Allen 1952, Lalucat et al. 2006).

Growth experiments of axenic algal and bacterial cultures

Total cell counts and dead cell counts of *Tetraselmis indica* in axenic and xenic cultures and in

axenic cultures in combination with each isolated bacterium were studied for a period of algal growth (Figs. 3 to 5). Bacterial cell concentrations in the xenic culture gradually increased with time, reaching a concentration of 32.8×10^6 on the 28th day after inoculation. A rapid decline in total cell counts was recorded in the axenic compared to xenic cultures in the senescent phase of algal growth (Fig. 3). In the senescent phase, the total algal cell counts in the axenic culture with either *Acinetobacter* sp. or *Ruegeria* sp. were relatively high (Fig. 4) and the dead cell counts showed a significant reduction (Fig. 5). This was in contrast to axenic culture alone and axenic culture with *Pseudomonas* sp., which showed lower total cell counts (Fig. 4) and increased dead cell counts (Fig. 5). Significant cell lysis and hence a decrease in total cell counts was observed in axenic cultures during the senescent phase, whereas no such lysis was observed in xenic cultures. The presence of bacteria in xenic cultures led to an increased specific growth rate of 0.20 d^{-1} compared to axenic cultures with a specific growth rate of 0.15 d^{-1} in the exponential phase. The presence of bacteria on the discarded cell walls of alga in the xenic culture was observed using SYBR Gold staining and epifluorescence microscopy (Fig. 6).

Carbohydrate utilisation (mono- and disaccharide)

In the control tubes, to which bacteria were not added, the carbohydrate substrate was not utilised and there was no subsequent change in the colour of the indicator and no evolution of gas, indicating a negative reaction. Some bacterial strains possess enzymes that degrade peptones present in the media to amino acids. These in turn are enzymatically converted by oxidative deamination into keto amino acids before being metabolised through the Krebs cycle for energy production. These reactions release ammonia, which accumulates in the medium, forming ammonium hydroxide and producing an alkaline environment, causing the colour of the media to alter from red to pink. Pink media was observed in the control tubes with no carbohydrates for *Pseudomonas* sp. and *Ruegeria* sp., indicating that these

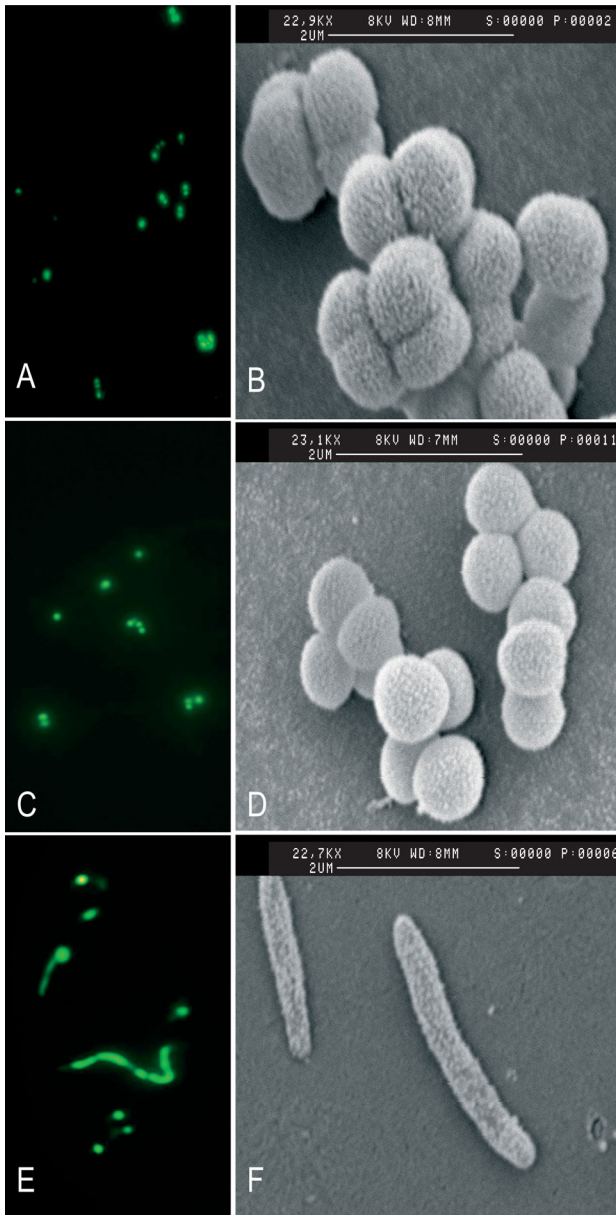


Fig. 2. *Acinetobacter* sp., *Pseudomonas* sp. and *Ruegeria* sp. Morphology of the isolated bacteria. (A,B) Epifluorescence and scanning electron microscopy (SEM) micrograph of *Acinetobacter* sp. showing bacteria present in pairs; (C,D) epifluorescence and SEM micrograph of *Ruegeria* sp. showing its spherical morphology; (E,F) epifluorescence and SEM micrograph of *Pseudomonas* sp. showing its rod-shaped morphology

enzymes were present. *Acinetobacter* sp. caused all the sugar solutions tested to turn yellow, indicating the formation of an acidic waste product following hydrolysis of the carbohydrate tested. The production of gas was observed only when sucrose was

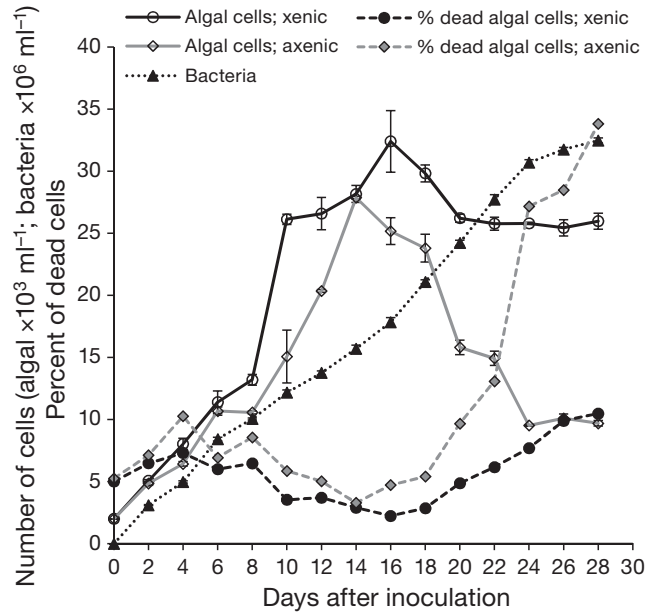


Fig. 3. *Tetraselmis indica*. Variation in total algal concentration, and percentage of dead algal cells in algal cultures without (axenic) and with (xenic) bacteria. Addition of a mixed community of bacteria (present in the original algal isolate) to the axenic algal cultures led to the increase in bacterial concentrations

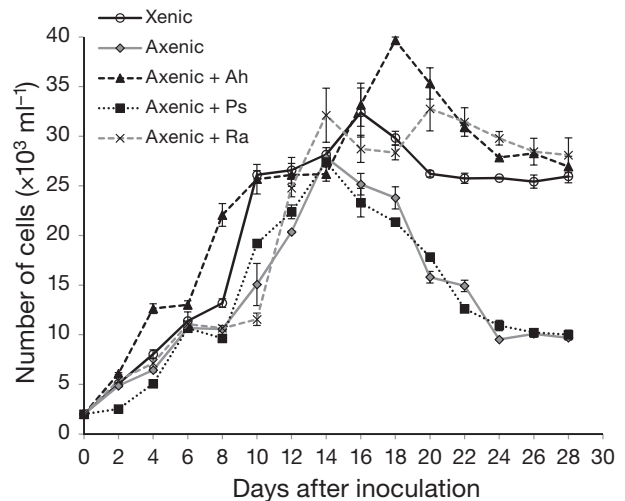


Fig. 4. *Tetraselmis indica*. Total algal cell concentrations in axenic and xenic cultures, and axenic cultures inoculated with each of the bacterial species isolated. Ps: *Pseudomonas* sp.; Ah: *Acinetobacter* sp.; Ra: *Ruegeria* sp. Values are means of triplicates \pm SE

utilised as the carbohydrate source (Fig. 7). *Pseudomonas* sp. and *Ruegeria* sp. were unable to utilise some of the tested carbohydrates (Table 1) and no gas production was observed by either of these 2 bacterial strains with any of the substrates.

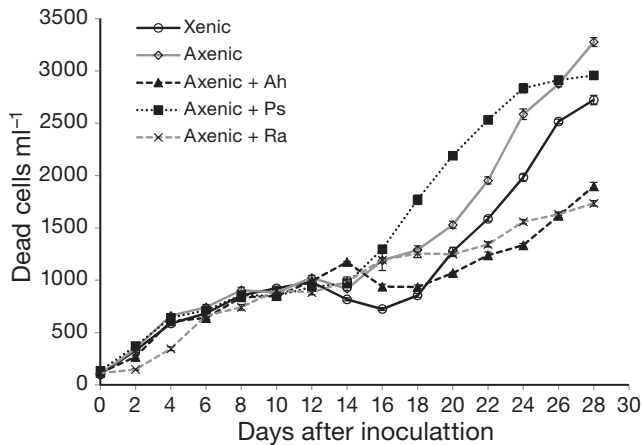


Fig. 5. *Tetraselmis indica*. Dead cell concentrations from the experiment described in Fig. 4 for axenic and xenic cultures, and axenic cultures inoculated with each of the bacterial species isolated. Ps: *Pseudomonas* sp.; Ah: *Acinetobacter* sp.; Ra: *Ruegeria* sp. Values are means of triplicates \pm SE

Evaluation of the biodegradability of polysaccharides

Pseudomonas sp. and *Acinetobacter* sp. showed carbohydrase activity and formed a halo-like precipitation zone (Fig. 8) with 4 (azo-galactan, azo-carob galactomannan, β -glucan and pectin) of the 8 carbohydrate substrates tested (Table 2), whereas *Ruegeria* sp. showed much less carbohydrase activity and only with pectin. Additionally, *Pseudomonas* sp. also demonstrated a low level of activity of the enzyme dextrinase.



Fig. 7. *Acinetobacter* sp. Utilisation of sucrose by the isolated bacteria. The tube on the left is the control and the tube of the right demonstrates the colour change in the media following utilisation of the sucrose and the production of gas in the inverted tube

DISCUSSION

This study has investigated the influence of bacteria on the cell growth and cell death kinetics of *Tetraselmis indica* under culture conditions. The study showed that in contrast to xenic culture, reduced cell growth and enhanced mortality occurred in axenic culture. Bacterial inoculation into cultures of *T. indica* had a pronounced effect on algal growth, which either decreased (as observed with *Pseudomonas* sp.) or increased (as observed with *Acinetobacter* sp.). Following inoculation with *Acinetobacter* sp. and *Ruegeria* sp., prolonged survival was observed. Bacterially regenerated carbon from the discarded cell walls was very likely utilised by the

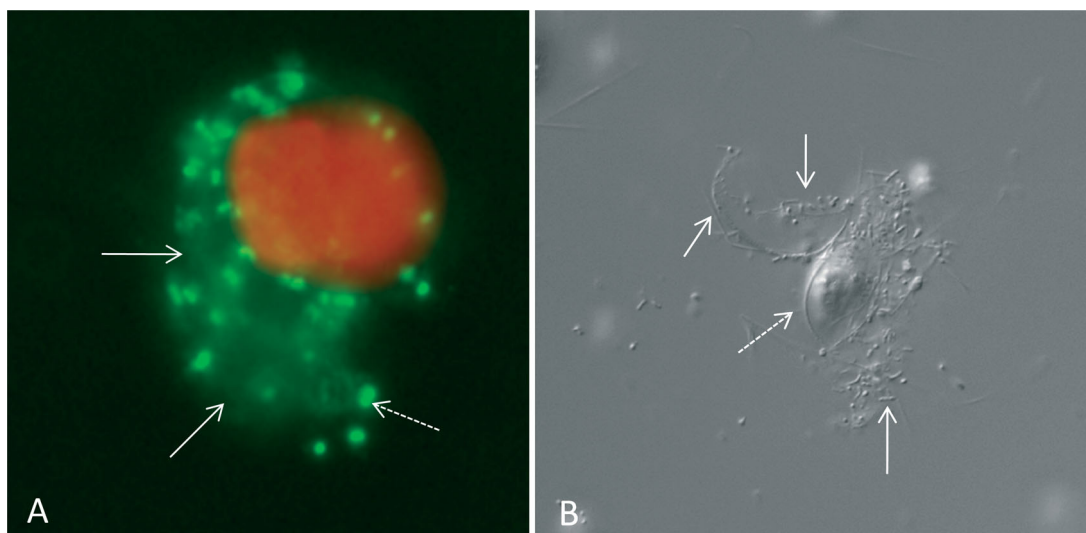


Fig. 6. *Tetraselmis indica*. (A) Epifluorescent micrograph showing the attachment of bacteria to the discarded cell wall of *T. indica*; solid arrows point towards the cell wall and the dotted arrow shows bacterial cells. (B) Phase contrast micrograph of the discarded and disintegrating cell wall showing the presence of bacteria; solid arrows point towards the disintegrating cell wall and bacteria and the dotted arrow shows a live algal cell

Table 1. *Acinetobacter* sp., *Pseudomonas* sp. and *Ruegeria* sp. Utilisation of different carbohydrates by the 3 bacterial species isolated. + indicates the sugar was utilised and – that the bacteria were unable to utilise it

Sugar	<i>Pseudomonas</i> sp.	<i>Acinetobacter</i> sp.	<i>Ruegeria</i> sp.
Sucrose	–	+ (with production of gas)	–
Dextrose	+	+	+
D-(+)-mannose	+	+	+
D-(+)-xylose	–	+	+
D-(+)-maltose	–	+	+
Lactose	–	+	–
D-(–)-mannitol	+	+	+
L-(+)-arabinose	+	+	+
D-(+)-galactose	+	+	+
N-acetyl glucosamine	–	+	+
D-glucuronic acid	–	+	+

cultures during the senescent phase, thereby prolonging the survival of the *T. indica* population. This reinforces the concept that bacteria can enhance the growth of algae.

Another interesting result was that the xenic culture, which contained both culturable and non-culturable bacteria, demonstrated less growth compared to that associated with *Acinetobacter* sp. alone. Therefore, there could be internal competition within the bacterial community that results in this reduced growth of the alga. In the xenic cultures, bacteria were found to be attached to the discarded

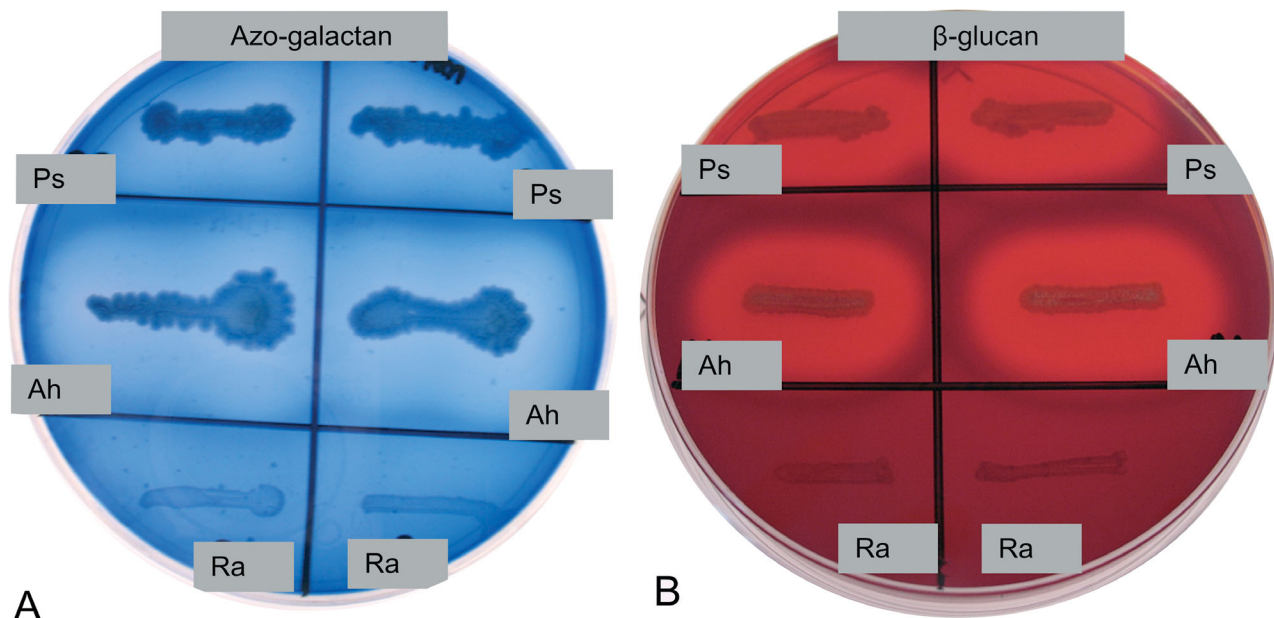


Fig. 8. *Acinetobacter* sp., *Pseudomonas* sp. and *Ruegeria* sp. Degradation of carbohydrates by the isolated bacteria. Ps: *Pseudomonas* sp., Ah: *Acinetobacter* sp., Ra: *Ruegeria* sp. (A) Activity of endo-1,4- β -galactanase when azo-galactan was the substrate, (B) activity of β -glucanase when β -glucan was the substrate

Table 2. *Acinetobacter* sp., *Pseudomonas* sp. and *Ruegeria* sp. Carbohydrate substrate utilisation by the 3 bacterial species isolated. – indicates no degradation of the substrate whilst + indicates degradation of the substrate

Carbohydrate (enzyme activity)	<i>Pseudomonas</i> sp.	<i>Acinetobacter</i> sp.	<i>Ruegeria</i> sp.
Azo-galactan (endo-1,4- β -galactanase)	+	+	–
Azo-carob galactomannan (β -mannanase)	+	+	–
β -glucan (β -glucanase)	+	+	–
Red pullulan (dextrinase)	+	–	–
Azo-CM-cellulose (cellulase)	–	–	–
Wheat arabinoxylan	–	–	–
Citrus pectin (pectinase)	+	+	+
AZ-rhamnogalacturonan (rhamnogalacturonanase)	–	–	–

cell walls (Fig. 6 and see the animation in Supplement 2 at www.int-res.com/articles/suppl/b015p061_supp/). The cell wall of *Tetraselmis* has been shown to be made of a pectin-like material, with galactose and galacturonic acid as major components (Lewin 1958, Gooday 1971). Pectins can consist of either a polymer of unsubstituted polygalacturonic acid residues or a backbone of galacturonic acid and rhamnose residues that are attached to galactan and arabinan side chains. Galacturonic acid and some other 2-keto sugar acids impart an acidic character to the cell walls of *Tetraselmis* (Becker et al. 1991, 1998). Experimental results of the current study on the bacterial utilisation of different carbohydrate substrates indicated which carbohydrate substrates these bacteria depend on for their survival. Bacterial strains were found to produce carbohydrases for degradation of polysaccharides generally present in the cell wall of *Tetraselmis* (glucans, galactans, galactomannans and pectins), whereas no such utilisation was observed for other wall substrates, such as cellulose, arabinoxylan and rhamnogalacturonan. The production of carbohydrases such as pectinases, glucanases, galactanases and mannanases from various species of *Pseudomonas* and *Acinetobacter* has also been reported in earlier studies (Katohda et al. 1979, Simonson et al. 1982, Braithwaite et al. 1995, 1997, Titapoka et al. 2008, Aboaba 2009, Zheng et al. 2011). Since *Acinetobacter* sp. and *Pseudomonas* sp. were able to utilise the same substrates at almost equal levels, as indicated by the carbohydrate utilisation results, this suggests that they may compete for the same substrates within the cell wall of *T. indica*. Importantly, when the alga was grown with only *Pseudomonas* sp. present, its growth was inhibited, leading to a prolonged lag phase. Growth resumed only when the algal culture was in its exponential phase, and became arrested again when the senescent phase was reached. Therefore, it can be speculated that *Pseudomonas* sp. has a negative influence on algal growth, notably it could be toxic for cell culture, and this bacterium is able to survive only as a competitor with the other associated bacteria in the cell wall environment. Even though *Ruegeria* sp. enhanced the survival of the alga after the senescent phase, there was a neutral effect on the culture growth during the lag phase. Hence, it can be concluded from this study that each bacterium associated with *T. indica* demonstrates a differential influence on algal growth.

As demonstrated in this study, these algae-associated bacteria are a potential source of useful carbohydrases and could be used to prevent the forma-

tion of biofilms and in applications involving biofilm dispersal due to their polysaccharide-degrading capabilities (Kaplan et al. 2003, Ramasubbu et al. 2005). In addition, *Acinetobacter* sp. could also be used in microalgal aquaculture to increase growth of algae. More widely, this study reinforces the ecological importance of bacteria in marine biogeochemical cycles, in hydrolysing organic matter and in influencing the dynamics of phytoplankton.

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