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Short communication

Characterisation of bacteria from the cultures of a *Chlorella* strain isolated from textile wastewater and their growth enhancing effects on the axenic cultures of *Chlorella vulgaris* in low nutrient media

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Running title: Bacteria enhance the growth of *Chlorella vulgaris*

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Highlights

1. **Bacteria associated with *Chlorella* sp. P02, isolated from open pond textile factory wastewater was found to be dominated by *Pseudomonas* and *Brevundimonas*.**
2. **The relative abundance of bacteria was different within *Chlorella* sp. P02 cultures in medium with varying nitrogen and phosphorus concentrations.**
3. **A diverse range of bacteria isolated from *Chlorella* sp. P02 were able to enhance the growth of an axenic variant of *C. vulgaris*, but only in low nutrient medium.**

Summary

There is increasing interest in the use of microalgae grown on wastewater to provide useful metabolites. Several bacteria have been shown to affect the growth rate and quality of the algae, but it is not clear if this is specific to a particular group of bacteria or if nutrient conditions can also influence this interaction. The bacterial community associated with a freshwater *Chlorella* sp. isolated from open pond textile factory wastewater was characterised and a diverse group of bacteria isolated. We provide evidence that nutrient concentrations affect bacterial community composition. When grown in BG11 medium, the community was dominated by *Pseudomonas* sp., but when grown in Chu 10 medium (which contains lower nitrogen and phosphorus), the relative abundance of a *Brevundimonas* spp. increased. Several of the bacteria isolated were able to influence the growth of an axenic *Chlorella vulgaris* culture. The *Pseudomonas* sp. had a negative effect in all media tested whereas several isolates enhanced *C. vulgaris* growth, but only in Chu 10 medium. This supports the theory that bacterial stimulation of algal growth is not limited to species-specific interactions but is influenced by environmental conditions. In low nutrient conditions, *Chlorella* sp. may be increasingly dependent on bacteria for growth.

1. Introduction

There is an increasing recognition that society needs to transition towards a circular economy. This is leading to a resurgence of interest in the idea of using microalgae grown on wastewater to provide useful metabolites including biofuels, nutraceuticals, pharmaceuticals and cosmetics (Christensen et al. 2011; Mohan et al 2016; Morales et al. 2019). This approach mitigates the prohibitive costs associated with nitrogen and phosphorus supply for algal growth as such nutrients are often in abundance in industrial wastewater (Craggs et al. 2011). Furthermore this method provides a ready supply of water in geographical areas where water sources are limited and acts to reduce the nutrient and toxic metal load of industrial wastewater released into the environment (Ryu et al. 2015). Several types of agro-industrial wastewater and sewage have been successfully used as a means of biomass production from large-scale microalgal culture (Ferrero et al. 2012; Hernández et al. 2013; Kim et al. 2014a), with specialised companies in operation in countries such as India, Australia and Germany. The resulting biomass can then be used to generate bioenergy (Biller et al. 2012).

The use of microalgae to clean wastewater usually involves either a single strain or a mixed community of microalgae that will adjust to the ambient abiotic/biotic conditions to form an established community, together with a consortium of bacteria. Certain species of bacteria have been shown to affect the growth rate and quality of the algae (Cho et al. 2015), can boost lipid production (Woertz et al. 2009; Cho et al. 2015) and can also aid in bio-flocculation, thus reducing the costs associated with harvesting biomass (Lee et al. 2013; Kim et al. 2014a). There are many reports of the growth enhancement properties microalgae, including *Rhodobacteriales* in the marine environment (Buchan et al. 2014; Amin et al. 2015), and *Burkholderiales*, *Caulobacterales, Rhizobiales*, *Rhodospirillales* and *Sphingomonadales* (Hayat et al. 2010; Krohn-Molt et al. 2013; Kumar and Gera 2014; Cecagno et al. 2015; Alves et al. 2016; Khan et al. 2017)in freshwater. In several cases this has been attributed to the production and exchange of nutrients beneficial for the

68 growth of the algae and/or the bacteria. This can include the exchange of vitamins for fixed carbon (
69 Kazamia et al. 2012), phytohormones (Geng and Belas, 2010; Amin et al. 2015) and also nutrients
70 such as nitrogen, sulphur and iron (Gonzalez-Bashan, 2000; Amin et al. 2009; Hernandez et al. 2009;
71 Amin et al. 2015; Cho et al. 2015; Torres-Monroy and Ullrich, 2018). There is also evidence that
72 bacteria can suppress the growth of potential pathogens. For example, *Nannochloropsis oculata* can
73 enhance the ability of *Roseobacter* clade bacteria (often found associated with this algae) to inhibit
74 the growth of the fish pathogen *Vibrio anguillarum* (Sharifah and Eguchi, 2011). However, not all
75 interactions will be beneficial. Algicidal bacterial species (typically of the Bacteroidetes or
76 Gammaproteobacteria such as *Altermonas*, *Pseudomonas* and *Pseudoaltermonas*, Meyer et al. 2017)
77 are frequently reported, with evidence to suggest that the nature of the interaction between algae
78 and bacteria is dependent on nutrient conditions (Geng and Belas, 2010). In addition, bacteria and
79 algae will compete for nutrients, with bacteria better able to scavenge phosphorus (Thingstad et al.
80 1993), but algae outcompeting bacteria for ammonia (Risgaard-Petersen et al. 2004).
81
82 Many of the reports of enhanced growth of microalgae by bacteria have indicated this to be a
83 species-specific interaction (Fukami et al. 1997; Sapp et al. 2007; Watanabe et al. 2005) or studied
84 only one bacterial species (Gonzales and Bashan, 2000; Kim et al. 2014b). However several dozen
85 bacterial species can be present within the consortium (Krohn-Molt et al. 2013; Cho et al. 2015) and
86 there is increasing evidence that several, diverse bacterial species are able to modify the growth of
87 algae (Cho et al. 2015; Kimbrel et al. 2019). It is also unclear whether environmental factors, such as
88 nutrient conditions, can also affect the composition of bacterial species present. Certain studies
89 have reported bacterial colonisation of microalgae to be species-specific rather than driven by
90 environmental factors (Grossart et al. 2005; Eigemann et al. 2013). Conversely, others have linked
91 changes to the community composition of algae-associated bacteria with changes to algal growth
92 phase (Jasti et al. 2005; Sapp et al, 2007) or nutrient conditions (Palacios et al. 2019; Kimbrel et al.

2019). A shift in nutrient conditions could also alter the balance from a mutualistic to a competitive interaction (Geng and Belas, 2010; Thingstad et al. 1993).

Chlorella sp. have been widely studied with respect to their interactions with bacteria, with reports of *Azospirillum*, *Flavobacterium*, *Hyphomonas*, *Rhizobium* and *Sphingomonas* enhancing growth, lipid content and flocculation (Gonzalez-Bashan, 2000; Kim et al. 2014b; Cho et al. 2015; Ferro et al. 2019). In the current study, we aimed to characterise the bacterial community associated with a freshwater *Chlorella* sp. isolated from an open pond textile factory wastewater in Chennai, India, and to determine whether the community differed within a selection of algal culture media containing high and low concentrations of nitrogen and phosphorus. We aimed to isolate members of the bacterial consortia present to determine which species influenced the growth (either positively or negatively) of an axenic *Chlorella* sp. under different nutrient conditions. Our *a priori* hypotheses were that a) nutrient conditions influence both the composition and the growth-promoting abilities of the bacteria present and b) growth promotion is not limited to a single bacterial species present.

2. Materials and Methods

2.1 Isolation and molecular identification of bacteria associated with *Chlorella* sp. P02

A *Chlorella* sp. P02 (NCBI accession number MF692949) was originally isolated and purified from an open pond textile factory wastewater in Chennai, India (provided by Dr. Sivasubramanian, Phycospectrum Environment Research Centre, Chennai, India). The alga was initially cultured in BG11 medium (Stanier et al, 1971) and bacteria isolated from this culture using BG11 amended with filter-sterilised culture supernatant from the *Chlorella* sp. P02, solidified with 1.5 % BactoAgar (BD Diagnostics, Oxford, UK) or 2 % Gelzan (Sigma-Aldrich, Dorset, UK). Agar plates were incubated in both the light and dark for three weeks before individual colonies were picked. Only one type of bacterial colony grew preferentially in the presence of light. But as several microbial types were

119 present in these colonies and they proved difficult to purify, these strains were not included in the
120 study. Bacterial isolates were identified by sequencing the V1–V3 region of the 16S rRNA gene using
121 the PCR primers 27F (AGRGTTTGATCMTGGCTCAG) (Vergin et al., 1998) and 519Rmod
122 (GTNTTACNGCGGCKGCTG) (Andreotti et al., 2011). This primer set was chosen to enable a match to
123 the bacterial 16S rRNA gene sequences obtained from the in depth sequencing of *Chlorella* sp. P02
124 cultures (i.e. non-cultured) below (section 2.2). The 50 µL reaction volume contained 10x PCR buffer
125 (Qiagen, Manchester, UK), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U of Taq DNA polymerase (Qiagen,
126 Manchester, UK), 0.5 µM of forward and reverse primers and a small section of bacterial colony
127 added to the PCR mix using a sterile pipette tip. PCRs were initially denatured for 3 min at 94 °C,
128 followed by 30 cycles of 94 °C for 30 s, primer annealing at 57 °C for 45 s and elongation at 72 °C for
129 60 s. A final elongation step was performed at 72 °C for 5 min. This was performed in triplicate for
130 each bacterial strain. No template controls were included for all PCR amplifications. PCR products
131 were cleaned using the cleaned using the QIAquick PCR purification kit (Qiagen, Manchester, UK)
132 and sent to DNA Sequencing and Services (Dundee, UK). Accession numbers for the bacterial strains
133 can be found using the numbers MF692941 - MF692948.
134
135 **2.2 Illumina MiSeq sequencing of bacterial community associated with *Chlorella* sp. P02**
136 DNA was extracted from triplicate *Chlorella* sp. P02 cultures using an AllPrep DNA/RNA Mini Kit
137 (Qiagen, Manchester, UK) following the instructions of the manufacturer. A partial fragment of the
138 16S rRNA gene was sequenced using the PCR primers and conditions above, with the exception of a
139 reduced number of amplification cycles (20). No template controls were included for all PCR
140 amplifications. The PCR products were cleaned using the QIAquick PCR purification kit (Qiagen,
141 Manchester, UK) and sent to MR DNA (www.mrdnalab.com, TX, USA). PCR products were then
142 subjected to a further five PCR cycles using primer sets modified with multiplexing identifier (MID)
143 adaptors for barcode tagging, thereby allowing for post-sequencing separation of the samples.
144 Following PCR, all amplicon products from different samples were mixed in equal concentrations and

purified using the Agencourt AMPure XP Purification System (Beckman Coulter, Bromley, UK). The pooled and purified PCR product was used to prepare DNA libraries by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed on a MiSeq following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, TX, USA) as follows: sequences were de-multiplexed, depleted of barcodes and primers, sequences <150 bp or with ambiguous base calls and with homopolymer runs exceeding 6 bp removed, denoised, operational taxonomic units (OTUs) generated (at 97% similarity) and chimeras removed. Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006). Sequence data can be found using the NCBI database accession number PRJNA401004. PERMANOVA was used to test for possible differences in the composition of the bacterial community present within the algal cultures grown on different media. Following on from this, one way ANOVA was used to determine significant differences in the relative abundance of key taxa within the different medium tested.

2.3 Co-incubation of axenic *Chlorella vulgaris* (CCAP 211/11B) with bacterial isolates

To determine if the isolated bacteria influenced the growth of *Chlorella* sp., repeated attempts were made to create an axenic version of the culture using combinations of antibiotics, UV treatments, sonication, plating onto agar and single cell sorting using a flow cytometer (Sensen et al. 1993; Guillard, 2005). Unfortunately, all attempts were unsuccessful and so an axenic *Chlorella vulgaris* was obtained (Culture Collection of Algae and Protozoa (Oban, UK) strain CCAP 211/11B). *C. vulgaris* CCAP 211/11B was isolated from a eutrophic freshwater pond near Delft, Netherlands in 1889.

Although a minimal medium, BG11 contains high concentrations of nitrate and phosphate (Table 1). The BG11 medium was therefore modified (LN BG11) to better reflect the lower nitrogen and phosphorus composition of textile wastewaters (Lim et al., 2010; El-Kassis et al. 2014) by reducing the nitrate ($NaNO_3$) concentration to 0.23 mM (BG11 contains 17.6 mM) and the phosphate

171 (K_2HPO_4) concentration to 0.045 mM (BG11 contains 0.23 mM). We also selected Chu 10, a low

172 nutrient medium containing 0.24 mM $Ca(NO_3)_2$ and 0.029 mM K_2HPO_4 (Chu et al. 1942) (Table 1).

173

Table 1

Nutrient composition in nutrient-replete (HN) and nutrient-limited (LN) media used in *Chlorella sp*. batch cultures.

BG11 Nutrient [mM]	**LN BG11** Nutrient [mM]	**Chu10** Nutrient [mM]
$NaNO_3$ [17.6]	$NaNO_3$ [0.23]	$Ca(NO_3)_2$ [0.24]
K_2HPO_4 [0.23]	K_2HPO_4 [0.045]	K_2HPO_4 [0.029]
$MgSO_4.7H_2O$ [0.3]	$MgSO_4.7H_2O$ [0.3]	$MgSO_4.7H_2O$ [0.1]
$CaCl_2.2H_2O$ [0.24]	$CaCl_2.2H_2O$ [0.24]	
Citric acid [0.031]	Citric acid [0.03]	
$(NH_4)_5[Fe(C_6H_4O_7)_2$ [0.021]	$(NH_4)_5[Fe(C_6H_4O_7)_2$ [0.021]	$FeCl_3$ [2.93 x 10^{-3}]
$EDTANa_2$ [2.7 x 10^{-3}]	$EDTANa_2$ [2.7 x 10^{-3}]	
Na_2CO_3 [0.19]	Na_2CO_3 [0.19]	Na_2CO_3 [0.19]
Trace metal solution¥	Trace metal solution¥	
		$Na_2SiO_3.5H_2O$ [0.2]

¥Contains per L: 2.86 g H_3BO_3; 1.81g $MnCl_2.4H_2O$; 0.22 g $ZnSO_4.7H_2O$; 0.39 g $Na_2MoO_4.2H_2O$; 0.08 g $CuSO_4.5H_2O$; 0.05 g $Co(NO_3)2.6H_2O$.

We compared the effect of each bacterial isolate on the growth of *C. vulgaris* in BG11, LN BG11 and Chu 10 media. Before each experiment commenced, the *C. vulgaris* culture was screened for the presence of bacteria by both microscopy and flow cytometry. Using a starting concentration of 1 x 10^5 *C. vulgaris* and approximately 2.5 x 10^7 washed bacteria (aiming for an alga: bacterium ratio of 1:250), the influence of each bacterial isolate on the growth of axenic *C. vulgaris* was first assessed using daily OD measurements ($OD_{750\ nm}$). Growth of *C. vulgaris* with the addition of bacteria was compared to growth of the axenic *C. vulgaris* without the addition of bacteria. Each assay was preformed using triplicate cultures and the whole experiment was repeated a further three times to confirm results. Results shown are from an individual experiment (using triplicate cultures). Further

191 experiments used flow cytometry to accurately monitor *C. vulgaris* and bacterial densities in cultures
192 with and without the addition of bacterial strain 113. Samples of culture (1 mL) from three replicate
193 culture were fixed with 50 µl of 50 % gluteraldehyde and stained with the DNA stain SYBR green
194 (Fisher Sceintific, Leicestershire, UK) for 1h then analysed using a FACSort flow cytometer (Becton
195 Dickinson, Oxford, England). Flow cytometer flow rate was calibrated (ca. 11 µL min^{-1}) and samples
196 were diluted if required to maintain counts below 1000 events sec^{-1}.
197
198
199 **3. Results and Discussion**
200 The 16S rRNA gene sequences of the bacterial isolates were compared to the composition of the
201 microbial community present within the BG11 culture, as obtained by in depth sequencing of
202 bacterial 16S rRNA genes (Table 2). The 16S rRNA gene sequences from the non-cultured DNA
203 extractions reveal that when grown in BG11, the *Chlorella* sp. P02 culture was dominated by
204 *Pseudomonads*, with *Caulobacterales* and *Rhodospirillales* also present in higher numbers (Table 2).
205 Culture-independent studies of the bacterial communities associated with *Chlorella* sp. have
206 previously identified several of these bacterial groups to be present, including members of the
207 *Actinomycetales, Burkholderiales, Caulobacterales*, *Rhizobiales*, *Rhodospirillales*, *Rhodobacterales*
208 and *Sphingomonadales* (industrial wastewater pond, Hamburg, Germany; Krohn-Molt et al. 2013;
209 swine wastewater pond, Korea; Cho et al. 2015). We were able to isolate representative members of
210 several of the bacterial groups present within the *Chlorella* sp. P02 culture, with the exception of the
211 *Rhodospirillales* and *Actinomycetales*. Of particular note was the high relative abundance of
212 *Pseudomonadale* 16S rRNA gene sequences (Table 2). The dominant *Pseudomonas* sp. strain isolated
213 from the *Chlorella* sp. P02 culture had several properties that may have influenced the isolation of
214 other less dominant bacteria. Firstly, this strain swarmed readily across the plate, rapidly out-
215 competing slower growing bacteria. Secondly, Burkholder diffusion assays (Burkholder et al. 1996),
216 used to assess the inhibition of growth in the presence of the *Pseudomonas* sp. isolate indicated all

other isolates were inhibited by the presence of the *Pseudomonas* sp. (results not shown). This ability of Pseudomonads to inhibit the growth of other bacteria has been previously reported, and this has been linked to the production of secondary metabolites including rhamnolipids and phenazine (Tedesco et al. 2016; Cardozo et al. 2013).

Growth in the different nutrient media significantly altered the relative sequence abundance of 16S rRNA gene sequences, indicating a shift in the balance of bacterial species resident within the *Chlorella* sp. P02 culture (Table 2; PERMANOVA Pseudo-F 6.4328; $p = 0.006$). Of note was a significant decrease in the relative abundance of 16S rRNA gene sequences affiliated to Pseudomonads and an increase in *Caulobacterales* in both LN BG11 and Chu 10 medium, and a slight but significant increase to the relative abundance of *Burkholderiales* in LN BG11 (Table 2). It is feasible that the *Brevundimonas* spp. were better adapted to the lower nutrient concentrations present within the LN BG11 and Chu10 media, out-competing the *Pseudomonas* spp. under these conditions despite the ability of the *Pseudomonas* sp. strain to inhibit the growth of the *Brevundimonas* sp.

We aimed to study the effect of our bacterial isolates on the growth of an axenic version of *Chlorella* sp. P02 but, as detailed above, were unable to make an axenic version. As a compromise, each bacterial strain was screened for their ability to alter the growth of axenic *C. vulgaris* based on OD obtained at stationary phase in BG11, LN BG11 and Chu 10 medium (Figure 1a). This particular strain was chosen as comparison of ribosomal internal transcribed spacer (ITS) sequences show it was a reasonable match to our *Chlorella* sp. P02 (Supplementary Figure 1) and we were satisfied that this strain was indeed axenic (via both microscopy (see Figure 1e) and flow cytometry). In addition, comparisons of bacterial strains reported to be associated with *Chlorella* sp. with those isolated within this study show many similarities (*e.g.* members of the *Pseudomonadales, Sphingomonadales*,

242 *Rhizobiales* and *Caulobacterales*; Lakaniemi et al. 2012; Toyama et al. 2018), suggesting that

243 *Chlorella* sp. tend to associate with particular groups of bacteria.

244

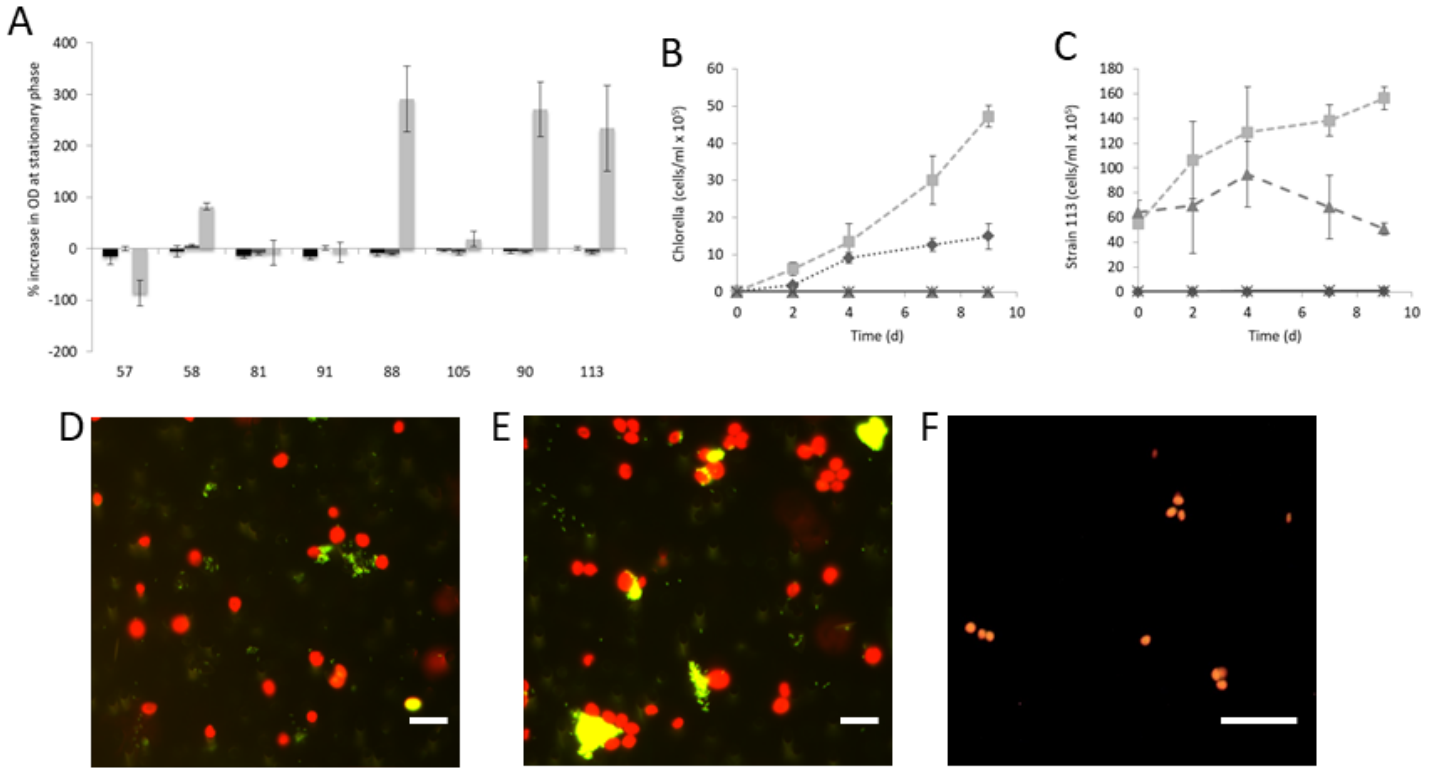
245

Table 2

Comparison of the relative abundance of bacterial groups associated with *Chlorella* sp. P02 (isolated from an textile factory wastewater open pond) grown in BG11, LN BG11 and Chu10 media. Relative abundance and composition of 16S rRNA genes was determined using 16S rRNA tagged Illumina MiSeq. One-way ANOVA was used to assess the taxonomic Orders that significantly differed in relative sequence abundance between media.Those showing significant differences ($p < 0.05$) are underlined. Also shown are the identities of bacteria that were isolated from the P02 culture with close sequence similarity to those identified within the Illumina MiSeq 16S rRNA dataset along with their NCBI accession numbers.

STRAIN	RELATIVE SEQUENCE ABUNDANCE				ISOLATED REPRESENTATIVES		
Order	BG11 (%) (± SD)	LN BG11 (%) (± SD)	Chu 10 (%) (± SD)	One-way ANOVA F (*p*)	Identity	Strain ID	Accession number
Pseudomonadales	89.1 (± 7.1)	78.9 (± 10.2)	30.3 (± 28.3)	9.3 (0.014)	*Pseudomonas* sp.	57	MF692946
Caulobacterales	3.6 (± 1.4)	16.2 (± 10.1)	41.6 (± 13.2)	12.1 (0.008)	*Brevundimonas* sp.	58	MF692945
Rhodospirillales	6 (± 5.1)	1.4 (± 1.7)	23.3 (± 20.8)	2.6 (0.153)	NONE ISOLATED		
Rhizobiales	0.9 (± 0.6)	3.4 (± 2.1)	4.5 (± 5.9)	0.8 (0.51)	*Bosea* sp.	81	MF692944
					Methylobacterium sp.	91	MF692941
Actinomycetales	0.2 (± 0.1)	0.02 (± 0.01)	0.1 (± 0.1)	2.7 (0.143)	NONE ISOLATED		
Rhodobacterales	0.07 (± 0.02)	0.06 (± 0.02)	0.06 (± 0.02)	0.2 (0.843)	*Catellibacterium* sp.	88	MF692943
Sphingomonadales	0.02 (± 0.004)	0.04 (± 0.004)	0.04 (± 0.004)	1.9 (0.23)	*Sphingomonas* sp.	105	MF692948
Burkholderiales	0.02 (± 0.007)	0.03 (± 0.001)	0.02 (± 0.002)	12 (0.008)	*Pseudoacidovorax* sp.	90	MF692942
					Hydrogenophaga sp.	113	MF692947

No bacterial isolate increased or decreased the OD of the axenic *C. vulgaris* culture at stationary phase in BG11 or LN BG11 at stationary phase (Figure 1a), indicating there were no impacts on growth. However, several bacterial isolates influenced the OD of axenic *C. vulgaris* in Chu 10 medium (Figure 1a). *Pseudomonas* sp. isolate 57 reduced the OD of *C. vulgaris* culture by 86.12 % (± 25 %); this growth-reducing property was not apparent in BG11 or LN BG11 media. In contrast, *Brevundimonas* sp. isolate 58, *Catellibacterium* sp. isolate 88, *Sphingomonas* sp. isolate 105, *Pseudoacidovorax* sp. isolate 90 and *Hydrogenophaga* sp. isolate 113 all increased the OD. In the case of isolate 90, an increase in OD of 291.3 % (± 63.8 %) was measured. The *Rhizobiales Bosea* sp. isolate 81 and *Methylobacterium* sp. isolate 91 had no impact on OD measurements. The growth-enhancing properties were confirmed by flow cytometry for axenic *C. vulgaris* and *Hydrogenophaga* sp. isolate 113. Both *C. vulgaris* (Figure 1b) and isolate 113 (Figure 1c) numbers were higher when co-cultured. When examined microscopically, it was apparent that a closer association of *C. vulgaris* and isolate 113 occurred within the Chu10 media with aggregates of algae and bacteria evident (Figure 1d and 1e), as has been reported previously (Cho et al. 2015; Samo et al. 2018).



268

269 **Figure 1**

270 Co-cultivation of axenic *C. vulgaris* with bacterial isolates, showing A) the effect of bacterial strains on the optical density (750 nm) of *C. vulgaris* stationary phase cultures

271 in BG11 (black bar), LN BG11 (mid grey bar) and Chu10 media (light grey bar). Flow cytometry was used to confirm *Hydrogenophaga* strain 113 enhances the growth of *C.*

272 *vulgaris* CCAP211/11B: (B) algal counts in flasks containing the axenic *C. vulgaris* (diamond), *C. vulgaris* and 113 (square), 113 (triangle) and the Chu10 media control (X). (C)

273 shows bacterial counts in the axenic *C. vulgaris* (diamond), *C. vulgaris* and 113 (square), 113 (triangle) and the Chu10 media control (X). Error bars are standard deviation (n

= 3). (D) and (E) show SYBR-green stained co-cultures of *C. vulgaris* and strain 113 in BG11 (D) where bacteria did not form aggregates with the alga and Chu10 (E) media where large aggregates of algae and bacteria formed (bar = 10 µm). In these images, *C. vulgaris* is red due to chlorophyll autofluorescence and bacterial cells are green. Also shown is axenic *C. vulgaris* (F) (bar = 50 µm).

279 Our data suggests that several bacteria isolated from *Chlorella* sp. P02 enhanced the growth of *C.*
280 *vulgaris* 211/11B. Many of these bacterial strains are known to exhibit plant growth properties
281 (Hayat et al. 2010; Kumar and Gera, 2014; Cecagno et al. 2015; Alves et al. 2016; Khan et al. 2017),
282 with several also able to promote the growth of microalgae. For example, *Brevundimonas* sp. have
283 been shown to promote the growth of *Chlorella ellipsoidea* (Park et al. 2008) and the culture lifetime
284 (or delayed death phase) of *C. vulgaris* NIES227 (Vu et al. 2010), whilst members of the
285 *Rhodobacterales* promote phytoplankton growth (Seyedsayamdost et al. 2010). We can only
286 speculate as to the underlying mechanisms involved in the growth promotion of *C. vulgaris* by the
287 bacterial strains isolated in this study. There are several ways that bacteria function to promote the
288 growth of algae. For example, there is evidence to suggest that alga-associated microbial
289 communities may be able to modulate the potency of algicidal compounds (Roth et al. 2008).
290 Bacteria may also facilitate nutrient uptake and/or synthesise compounds needed for growth
291 (Gonzalez-Bashan, 2000; Amin et al. 2009; Hernandez et al. 2009; Amin et al. 2015; Cho et al. 2015;
292 Torres-Monroy and Ullrich, 2018). A detailed study of metabolic interactions between a diatom and
293 *Sulfitobacter* sp. showed that the bacterium excretes the phytohormone indole-3-acetic acid using
294 diatom-synthesised tryptophan as a pre-cursor (Amin et al. 2015). Similar to the co-culture of the
295 axenic *C. vulgaris* and our isolate 113 (Figure 1b and 1c), growth of both the diatom and bacterium
296 were enhanced in co-culture.
297
298 The finding that growth promotion was apparent only in Chu10 medium implies the change in
299 nutrient regime may be responsible for an increased reliance on bacteria to provide nutrients
300 necessary for growth; Chu10 medium had the highest N:P ratio (Table 1)). Bacteria tend to be better
301 scavengers for P, especially when in low concentration, whereas at high P, algae will tend to
302 dominate (Currie and Kalff 1984; Thingstad et al. 1993). Another possibility is that the *Chlorella* cells
303 were 'leaking' more organic carbon under the low nutrient levels, a common response of
304 phytoplankton grown under conditions of N or P limitation in batch culture experiments (reviewed

in Thornton, 2014), thereby promoting mutualistic benefits (Brussaard et al. 1997). Chu 10 medium also has a lower proportion of iron and no trace metals (Table 1), potentially increasing the reliance of the algae on the bacteria present to provide trace nutrients through, for example, the efficient regeneration of algal organic matter or by the superior mechanisms of nutrient uptake utilised by bacteria. For example, in the marine environment, the uptake of scarce iron by bacteria can be facilitated by excretion of siderophores, small organic molecules with an exceptional affinity for iron. Phytoplankton associated *Marinobacter* sp. produce vibrioferrin, a compound that forms an iron complex that is highly photolabile. In the dark, vibrioferrin is used to solely supply the *Marinobacter* sp. with iron but under light conditions, inorganic soluble iron is released allowing uptake by both the *Marinobacter* and phytoplankton (Amin et al. 2009). In return, the *Marinobacter* sp. receives a supply of DOC (Amin et al. 2009).

Curiously, the *Rhizobiales* strains *Bosea* sp. 81 and *Methylobacterium* sp. 91 had no impact on the OD of the axenic *C. vulgaris* in our study, yet there are many reports of the plant and algal growth enhancing properties of these strains (Hayat et al. 2010; Kim et al. 2014b; Ferro et al. 2019). The low nutrient conditions provided by the Chu10 medium may also have been a factor here.

In conclusion, we have shown that bacteria associated with a *Chlorella* sp. isolated from a textile wastewater pond are capable of promoting growth of an axenic *Chlorella vulgaris* strain in very low nutrient media. The fact that several, diverse bacteria had a similar effect supports the theory that bacterial-induced algal growth promotion is not limited to species-specific interactions. However, growth stimulation only occurred within very low nutrient media, highlighting the possibility that a shift in nutrient regime can increase the dependence of algae on bacteria for growth. In low nutrient, or P limited wastewater, such as that produced by the textile industry, where algae are used to remove dyes from wastewater, algae may be increasingly dependent on bacteria for growth.

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Competing Interests Statement

None declared

Statement of informed consent

No conflicts, informed consent, human or animal rights applicable

Author contributions

Authors of this study contributed the in the following areas: conception and design of study: Tait, White and Llewellyn; acquisition of data: all authors; analyses of data: Tait, Kimmance, Tarran; original draft preparation review and editing: Tait, Llewellyn, White, Kimmance, Tarran.

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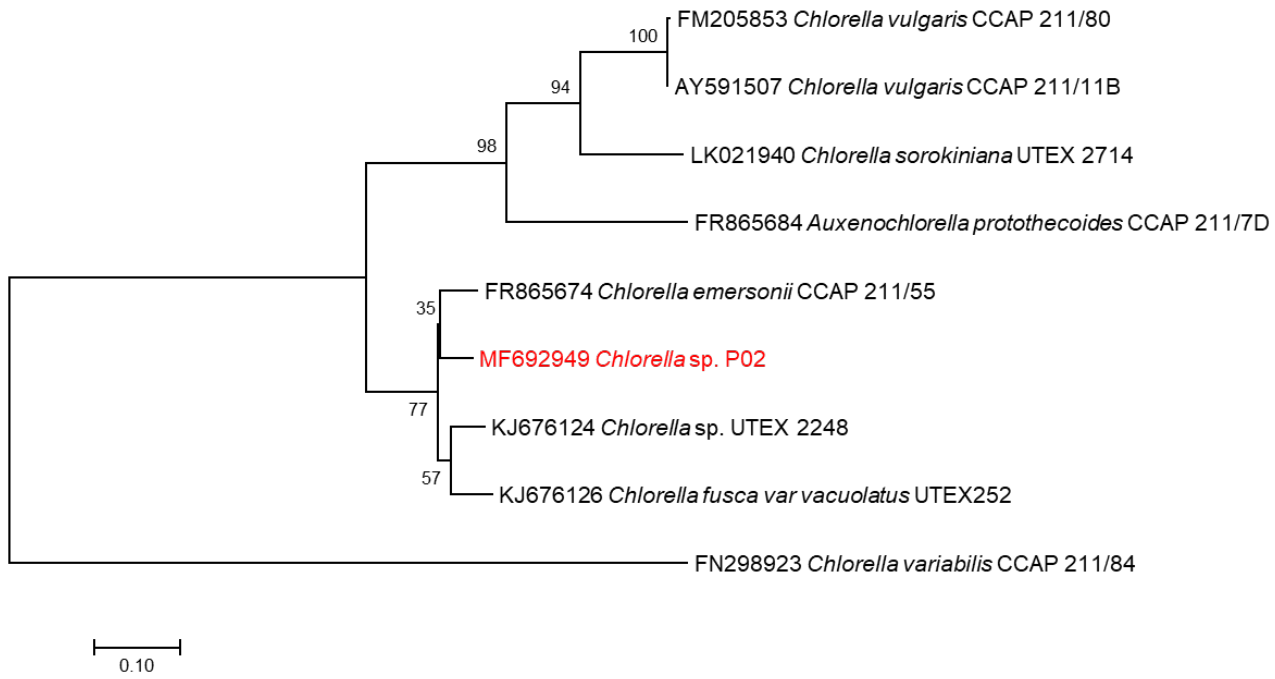
Supplementary Figure 1



Comparison of the ribosomal internal transcribed spacer sequence of *Chlorella* sp. P02 with other *Chlorella* sp., including CCAP 211/11B the axenic variant used within this study. The tree topology is based on maximum likelihood and bootstrap analysis was performed with 1,000 replications (MEGA7).