microbial biotechnology

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Different co-occurring bacteria enhance or decrease the growth of the microalga *Nannochloropsis* sp. CCAP211/78

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Summary

Marine photosynthetic microalgae are ubiquitously associated with bacteria in nature. However, the influence of these bacteria on algal cultures in bioreactors is still largely unknown. In this study, eighteen different bacterial strains were isolated from cultures of *Nannochloropsis* sp. CCAP211/78 in two outdoor pilot-scale tubular photobioreactors. The majority of isolates was affiliated with the classes *Alphaproteobacteria* and *Flavobacteriia*. To assess the impact of the eighteen strains on the growth of *Nannochloropsis* sp. CCAP211/78, 24-well plates coupled with custom-made LED boxes were used to simultaneously compare replicate axenic microalgal cultures with addition of individual bacterial isolates. Co-culturing of *Nannochloropsis* sp. CCAP211/78 with these strains demonstrated distinct responses, which shows that the technique we developed is an efficient method for screening the influence of harmful/beneficial bacteria. Two of the tested strains, namely a strain of *Maritalea porphyrae* (DMSP31) and a *Labrenzia aggregata* strain (YP26), significantly enhanced microalgal growth with a 14% and 12% increase of the chlorophyll concentration, respectively, whereas flavobacterial strain YP206 greatly inhibited the growth of the microalga with 28% reduction of the chlorophyll concentration. Our study suggests that algal production systems represent a 'natural' source to isolate and study microorganisms that can either benefit or harm algal cultures.

Received 7 October, 2020; revised 12 February, 2021; accepted 15 February, 2021.

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Microbial Biotechnology (2021) **14**(3), 1159–1170
doi:10.1111/1751-7915.13784

Funding Information

Jie Lian would like to thank China Scholarship Council (Grant Number: 201406310023). Part of this work was funded by European Union's Horizon 2020 Marie Skłodowska-Curie ITN EATFISH (Grant Number: 956697)

Introduction

Microalgae show great potential in producing numerous sustainable bioproducts as alternatives to fossil feedstocks (Ruiz *et al*., 2016; Wijffels and Barbosa, 2010; Berthold *et al*., 2019). A long-neglected aspect in algal biomass production is the role of bacteria that are co-occurring in algae cultivation systems (Cho *et al*., 2014; Biondi *et al*., 2018). Algal cultures are axenic in only a few applications, whereas all microalgae mass production systems inevitably contain a number of non-target organisms (contaminants), including bacteria (Zittelli *et al*., 2013; Newby *et al*., 2016). Bacteria are introduced in algae cultivation systems as algae stocks used as starter cultures are often not axenic (Biondi *et al*., 2017; Biondi *et al*., 2018; Gouveia *et al*., 2019). On the other hand, bacterial contaminants may enter cultivation systems through multiple operation processes, such as the supplementation of unsterilized medium or simply as airborne invaders in open algal cultures.

Microalgae–bacteria interactions are prevalent in natural aquatic environments, where microalgae release exudates into the phycosphere, the region immediately surrounding individual cells. Chemotaxis drives multiple bacteria to the phycosphere (Smriga *et al*., 2016), and metabolites are readily exchanged between algae and bacteria (Seymour *et al*., 2017). Although the phycosphere represents only a tiny area that can be as small as 1 μm surrounding the algal cell, it represents the hotspot for most of the algal–bacterial interactions that can profoundly affect the productivity and stability of aquatic ecosystems (Amin *et al*., 2012; Seymour *et al*., 2017).

Recent research on algal–bacterial interactions has usually been centred around the competitive or antagonistic aspects, which often involve competition for nutrients (Liu *et al*., 2012; Wang *et al*., 2016; Le Chevanton *et al*., 2016) or algicidal activities (Paul & Pohnert, 2011; Seymour *et al*., 2017). For instance, in a microcosm experiment it was found that bacteria were more efficient

than algae in the uptake of phosphorus (Liu *et al*., 2012). The advantage for bacteria is especially evident under phosphorus-limiting conditions (Zubkov *et al*., 2007). Apart from competing for nutrients with algae, some bacteria are known to inhibit algal cell division (van Tol *et al*., 2017) or cause algal cell lysis via secretion of algicidal compounds (Seyedsayamdost *et al*., 2011; Wang *et al*., 2012; Zheng *et al*., 2013).

In contrast to early views that bacteria mostly affect microalgae negatively, it has been demonstrated that mutualistic relationships between microalgae and bacteria are also prevalent, or even more common than antagonistic interactions (Seymour *et al*., 2017; Lian *et al*., 2018). Proof has been found from frequent observations that the absence of bacteria in algal cultures negatively affects algal physiology and growth (Bolch *et al*., 2011; Windler *et al*., 2014). In exchange for dissolved organic matter from microalgae, bacteria fix nitrogen (Foster *et al*., 2011; Thompson *et al*., 2012) and synthesize a wide range of molecules, including vitamins (Xie *et al*., 2013; Grant *et al*., 2014), the growth-promoting hormone indole-3-acetic acid (Amin *et al*., 2015; Dao *et al*., 2018) and the siderophore vibrioferrin (Amin *et al*., 2007; Lupette *et al*., 2016). The division of labour and close cooperation enables the holobiont to better adapt to and grow in changing aquatic environments, which has also triggered a growing interest for applications in industrial settings (Hom *et al*., 2015; Lutzu and Turgut Dunford, 2018; Yao *et al*., 2019).

Contrary to extensive tests of effects of environmental and chemical factors (irradiation, temperature, pH, nutrients, etc.) on algal growth in industrial photobioreactors, only a few studies have considered the effects of biotic factors such as associated bacteria. In order to assess the effects of co-occurring bacteria on microalgae in algal cultivation systems, we isolated and characterized bacteria from two pilot-scale outdoor tubular photobioreactors. Subsequently, a 24-well plate-based co-cultivation device was used to evaluate algal growth with addition of the isolated bacterial strains to axenic microalgae. Effects of bacteria on microalgae were further tested on a double-layer agar plate to verify algal–bacterial interactions.

Results and discussion

Bacterial isolation and identification

In order to recover as many different bacteria as possible from outdoor bioreactors, eight carbon sources were used for bacterial isolation. In total, we picked and sequenced 138 bacterial isolates from four samples from two outdoor photobioreactors with *Nannochloropsis* sp. CCAP211/78. All isolated bacteria were classified as *Proteobacteria* or *Bacteroidetes* and encompassed sixteen genera (Table S2). Two bacteria, closely related to *Celeribacter* sp. and *Maritalea porphyrae*, were the most frequently isolated and were recovered from all media (Table S1). Six bacteria were recovered from multiple media, while ten bacterial strains were recovered from only one medium. From medium YP (yeast and peptone extract), more bacterial species (11 out of 18) were recovered than from any of the other carbon sources also because many more colonies were obtained and picked (43 out of 138) from agar plates with YP.

We then chose 18 representative bacterial isolates for co-cultivation experiments. Of the 18 isolates, 11 belong to the class *Alphaproteobacteria* and five to *Flavobacteriia*. In addition, single isolates were obtained from the classes *Cytophagia* and *Saprospiria* (Table 1; Fig. S4). At the family level, isolates were mainly classified into three families: *Hyphomicrobiaceae*, *Rhodobacteraceae* and *Flavobacteriaceae*. It has also been corroborated by global surveys that phytoplankton-associated bacterial communities are often restricted to only a few bacterial classes including *Alphaproteobacteria* (*Rhodobacteraceae*), *Gammaproteobacteria* (*Alteromonadaceae*) and *Flavobacteriia* (*Flavobacteraceae*) (Amin *et al*., 2012; Teeling *et al*., 2012; Goecke *et al*., 2013; van Tol *et al*., 2017). Within *Alphaproteobacteria*, bacteria from the family *Rhodobacteraceae* are frequently associated with algae, of which the most studied ones are *Phaeobacter gallaeciensis* (Seyedsayamdost *et al*., 2011), *Dinoroseobacter shibae* (Wang *et al*., 2015), *Sulfitobacter* sp. (Amin *et al*., 2015) and *Ruegeria pomeroyi* (Durham *et al*., 2015). These apparently widespread patterns imply that the lifestyle of some bacteria within these groups is substantially related to that of algae.

When Sanger-sequenced 16S ribosomal RNA (rRNA) genes of the bacterial strains were compared to the 138 operational taxonomic units (OTUs) present in the four original bioreactor cultures, fourteen out of 18 bacterial strains had an identical match with OTUs encountered in the reactors, while four isolates had not (Table 1). The cultivable bacteria isolated in this study accounted for approximately 10% of the total OTUs (14 of 138) present in the original photobioreactor samples and represented nearly 7% of the total reads (11 820 of 152 260) in the bioreactor samples. Thus, a substantial fraction of bacteria in algal cultures remained uncultured under the conditions applied in our experiment. We observed sixteen OTUs with high relative abundance ($\geq$ 5%) in our algal cultures (Table S2), of which four (OTU533, 579, 327, 331) were successfully cultured. It is noticeable that although *Gammaproteobacteria* was one of the most abundant classes in two of four bioreactor cultures based on cultivation-independent assessment of bacterial diversity, no strains belonging to this class were recovered (Table S2 and Fig. S2).

Table 1. Bacterial strains isolated from *Nannochloropsis* cultures.

Strain	Accession Number (bacterial isolate)	Class	Family	Blast result[a]	Identity [%]	Accession number (Genbank best hit)	OTUs in bioreactors[b]	Identity [%]
GLU107	MH843917	*Alphaproteobacteria*	*Erythrobacteraceae*	*Porphyrobacter sanguineus*	100	LC349792	OTU247	100
PRO103	MH843918	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Algimonas arctica*	98	NR_137369	OTU321	100
DMSP31	MH843919	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Maritalea porphyrae*	99	AB583776	OTU327	100
DMSP20	MH843920	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Maritalea* sp.	99	AB758563	OTU331	100
PRO34	MH843921	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Maritalea* sp.	96	KP301112	OTU343	100
YP210	MH843922	*Alphaproteobacteria*	*Phyllobacteriaceae*	*Pseudohoeflea suaedae*	100	LT600545	OTU490	100
YP18	MH843923	*Alphaproteobacteria*	*Rhodobacteraceae*	*Celeribacter* sp.	100	MF045112	OTU582	100
YP26	MH843924	*Alphaproteobacteria*	*Rhodobacteraceae*	*Labrenzia aggregata*	100	MG273739	OTU247	100
YP29	MH843925	*Alphaproteobacteria*	*Rhodobacteraceae*	*Roseovarius mucosus*	99	CP020474	OTU585/709	100
YP202	MH843926	*Alphaproteobacteria*	*Rhodobacteraceae*	*Sulfitobacter* sp.	99	KY272045	OTU143/289	100
PAL103	MH843927	*Alphaproteobacteria*	*Sphingomonadaceae*	*Sphingorhabdus* sp.	99	KT325114	OTU259	98
DMSP2-Y	MH843928	*Cytophagia*	*Cytophagaceae*	*Emticicia* sp.	99	KP265953	OTU574	100
YP206	MH843929	*Flavobacteriia*	*Flavobacteriaceae*	*Aquaticitalea lipolytica*	99	NR_149769	OTU532/533	94
ALG110	MH843930	*Flavobacteriia*	*Flavobacteriaceae*	*Arenibacter* sp.	98	JX529985	OTU582	100
PAL10	MH843931	*Flavobacteriia*	*Flavobacteriaceae*	*Cellulophaga lytica*	100	MG456766	OTU519	96
PAL110	MH843932	*Flavobacteriia*	*Flavobacteriaceae*	*Maribacter* sp.	99	KT731371	OTU525	96
SUC105	MH843933	*Flavobacteriia*	*Flavobacteriaceae*	*Muricauda* sp.	99	KJ188010	OTU512	100
PRO13	MH843934	*Saprospiria*	*Saprospiraceae*	*Phaeodactylibacter xiamenensis*	99	NR_134132	OTU579	100

a. The best hit (highest per cent identity) in NCBI Genbank.
b. The best hit of photobioreactor OTUs.

Effect of bacteria on the growth of algae

To examine potential interactions between *Nannochloropsis* and the bacterial isolates, the bacterial isolates were re-introduced to axenic microalgae. All the cultures except the ones supplemented with strain YP206 had a similar growth pattern, that is, after rapid growth for nearly 5 days, the stationary phase was reached, which continued until the end of the experiment at day 11 (Fig. S3). No significant difference was found in relative fluorescence between axenic and non-axenic control cultures of *Nannochloropsis*. Addition of bacteria to the axenic *Nannochloropsis* sp. cultures had no significant impact on algal growth rates in the first six days (Fig. S3), except for YP206 where the growth rate was significantly lower (Fig. 1B), but mostly resulted in a slight decrease of the maximal fluorescent intensity reached at the stationary phase (Fig. 1A and Fig. S3).

For strain YP206 (*Flavobacteriia*), *Nannochloropsis* growth was strongly inhibited, leading to a reduction by more than 28% in fluorescent intensity. *Flavobacteriia* have repeatedly been reported to have antagonistic relationships with algae. For instance, *Kordia algicida* was shown to excrete an extracellular protease to lyse algal cells to acquire their dissolved organic carbon (Paul and Pohnert, 2011), and *Croceibacter atlanticus* was observed to release an unidentified molecule to arrest diatom cell division and increase secretion of organic carbon (van Tol *et al*., 2017). The closest relative of strain YP206 is *Aquaticitalea lipolytica* (99% identity of the 16S rRNA gene) that was isolated from Antarctic seawater and known to hydrolyse lipids (Xamxidin *et al*., 2016). However, when YP206 was co-cultured on agar plates with *Nannochloropsis*, the growth inhibition observed in liquid culture was not observed (Figs 1 and 2). Although mechanistic insight requires future research, one can speculate that the incubation time (7 days) used in the agar plate experiments described here was too short or that the algal density was still too low on the agar plate for the bacterial inhibition to take place, as some algicidal bacteria have been shown to only kill senesced algal cells in the stationary phase or decline phase (Seyedsayamdost *et al*., 2011; Wang *et al*., 2015). This has previously been explained by competition for limiting nutrients such as nitrogen (Meseck *et al*., 2006) and phosphorus (Danger *et al*., 2007; Liu *et al*., 2012). However, that is not likely to be the case for our results as nitrogen and phosphorus concentrations

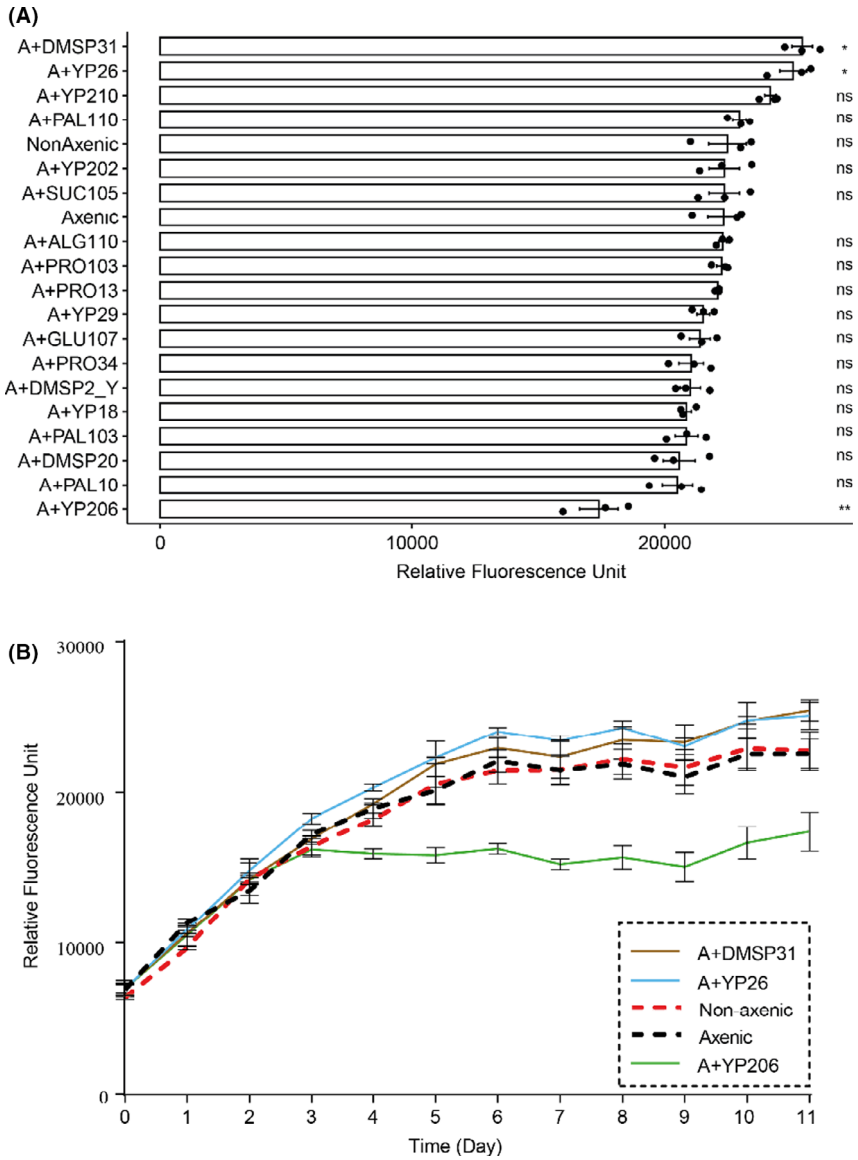

Fig. 1. Relative Fluorescence (~ algal biomass) of *Nannochloropsis* sp. CCAP211/78 co-cultured with individual bacterial strains.
A. Relative Fluorescence Unit (RFU) for *Nannochloropsis* sp. CCAP211/78 was calculated as maximal fluorescent intensity and compared to RFU of the axenic culture. Error bars represent standard deviation. Results of the statistical analysis are indicated by NS (*P*.adjust $>$ 0.05), * (*P*.adjust $\leq$ 0.05), and ** (*P*.adjust $\leq$ 0.01), respectively. The statistical results of pair-wise comparison against non-axenic culture (not shown) are the same as for the comparison to the axenic culture.
B. Growth curves of *Nannochloropsis* sp. with bacteria that significantly affected the growth (DMSP31, YP26 and YP206) and the axenic and non-axenic controls.

added would support much higher algae concentrations than those present in the stationary phase, and for nitrogen, it was confirmed in the stationary phase that it was not depleted (data not shown). Alternatively, release of toxic compounds by bacteria could contribute to the inhibitory effects observed at stationary phase (Fukami *et al.*, 1997; Mitsutani *et al.*, 2001). Many bacteria belonging to the family *Flavobacteriaceae* are able to glide on solid surfaces and decompose agar (Nedashkovskaya *et al.*, 2004). PAL10 and PAL110 displayed these features and formed larger and concave colonies on the agar surface (Fig. 2). Although both strains showed no significant effects on algal growth in liquid co-culture, they apparently enhanced the growth of *Nannochloropsis* sp. in the agar plate assay (Fig. 2). A possible explanation for the growth promotion on solid media could be that *Nannochloropsis* cells consumed the by-products from the agar degradation by the bacteria. For instance, *Cellulophaga lytica* (PAL10) has previously been shown to synthesize different kinds of

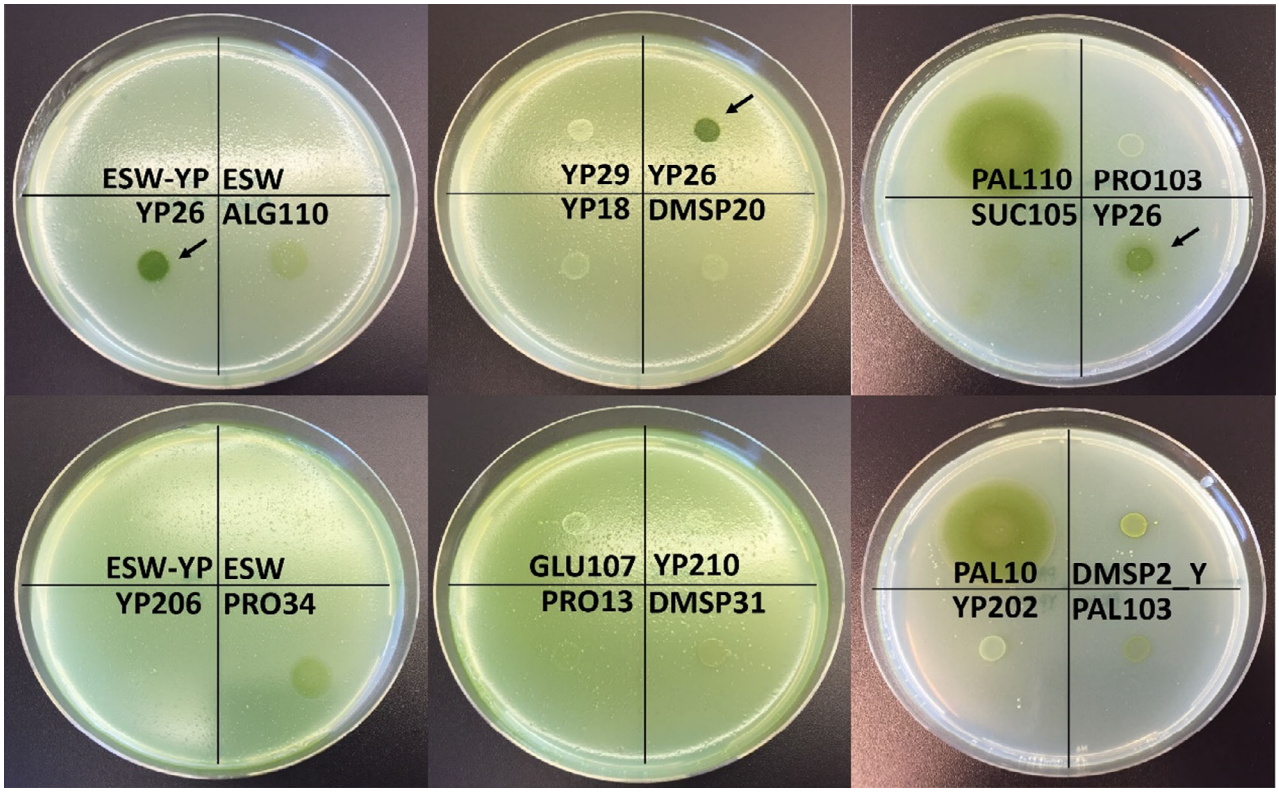

Fig. 2. Co-cultivation of *Nannochloropsis* sp. CCAP211/78 and bacterial strains on double-layer agar plates after seven days. ESW (Enriched natural seawater medium) and ESW-YP (ESW medium with peptone and yeast extract) were used as controls. The labelled names referred to the added bacteria. YP26 was added on three different plates as replicates indicated by arrows.

agarases (Lee and Choi, 2017), and the enzymatic hydrolysis of agar yields monomeric sugars, such as D-galactose, 3,6-anhydro-L-galactose and L-galactose-6-sulphate (Chi *et al*., 2012). Research has shown that supplementation with galactose increases the growth rate of *Nannochloropsis salina* by nearly 10% (Velu *et al*., 2015).

In contrast to the inhibition of algal growth by YP206, two bacterial strains (YP26, DMSP31) resulted in significantly better growth of the algae, and the intensity of algal fluorescence increased by 12–14% compared to the axenic control (Fig. 1). Members of the genus *Labrenzia* (YP26) have been isolated from a wide range of habitats and found to be frequently associated with other marine organisms (Weber and King, 2007; Coates and Wyman, 2017). These organisms include invertebrates such as molluscs, corals and sponges, and a wide variety of photosynthetic partners including seaweeds, diatoms, dinoflagellates, green and red algae (Boettcher *et al*., 2000; Groben *et al*., 2000; Sfanos *et al*., 2005; Weber and King, 2007). *Labrenzia aggregata* has also been isolated previously from *Nannochloropsis oculata* and *Nannochloropsis gaditana* (Han *et al*., 2016). A recent study revealed that *Labrenzia* sp. increased the biomass accumulation of the marine microalga *Isochrysis galbana* by 72% and the growth rate by 18% (Sandhya and Vijayan, 2019). On the other hand, it has been reported that a bacterial isolate (KD531) with 100% similarity to the partial 16S rRNA gene of our *Labrenzia aggregata* isolate had an algicidal effect on *Chlorella vulgaris* (Chen *et al*., 2014). The addition of bacterial lysate of KD531 to *Chlorella vulgaris* cultures caused nearly 20% reduction in biomass dry weight and nearly 60% reduction in lipid content. The contradiction between these and our observations may be due to strain-specific differences between isolates of *L. aggregata*, and/or different interactions of the bacterium with different algal hosts. Prior research has shown that some bacteria that are mutualistic to their native algal partner can be parasitic to foreign algae, which hints at co-adaption and evolution of algae and their associated microbiome (Sison-Mangus *et al*., 2014). In addition, we added live bacteria rather than a bacterial lysate, which may lead to a different effect. *Nannochloropsis* sp. also appeared to grow faster and denser on a solid growth medium in the presence of *Labrenzia aggregata* (Fig. 2). Agar plates have been the most commonly used method to study algal–bacterial interactions (Kazamia *et al*., 2012; Hertweck *et al*., 2017). For example, the vitamin B_{12}-dependent microalga *Lobomonas rostrata* could grow on agar plates only when vitamin B_{12} or a vitamin

B_{12}-synthesizing bacterium (*Mesorhizobium loti*) was added (Kazamia *et al.*, 2012). Therefore, it is tempting to speculate that the growth promotion observed here for *Labrenzia aggregata* could be related to inorganic nutrient exchange or algal acquisition of growth factors released by bacteria.

Although the growth increase of *Nannochloropsis* in the presence of *Maritalea porphyrae* (DMSP31) was significant in liquid cultures, this beneficial effect disappeared on the agar plate. It is interesting to note that *Maritalea porphyrae* (DMSP31) has been previously isolated from the thalli of the red alga *Pyropia yezoensis* (Fukui *et al.*, 2012). However, experimental evidence showed that these bacteria exhibited no apparent morphogenetic effects on the red alga (Fukui *et al.*, 2014), and therefore, the nature of a symbiotic relationship – if any – with the phototroph remains unknown. Some other bacterial isolates for which we did not find a significant effect have been previously associated to microalgae. For example, the family *Saprospiraceae* (strain PRO13) was the most prevalent taxon and also the most abundant one in industrial cultures of *Nannochloropsis salina* (Fulbright *et al.*, 2018). In addition, the 16S rRNA gene of strain PRO13 was identical to OTU579 found in the outdoor photobioreactors, particularly in sample HD0105 where this bacterium made up nearly 25% of the whole bacterial community (Table S2). In spite of this strikingly high relative abundance, co-culturing with strain PRO13 had no significant effect on the growth of *Nannochloropsis* sp. either in liquid co-cultures or on agar plates (Figs 1 and 2). Similarly, the study by Fulbright *et al.* (2018) reported that there was no correlation between the abundance of *Saprospiraceae* and growth of *N. salina*. However, the prevalence of this bacterium suggests it may have other functions in algal cultures, and the lytic capability of members of this bacterial family may relate to degrading cell debris for nutrient recycling (Fulbright *et al.*, 2018). DMSP2-Y is closely related to *Emticicia* sp., and species from the genus *Emticicia* have been recorded to live with *Chlorella vulgaris* (Otsuka *et al.*, 2008) and the macroalga *Cladophora glomerata* (Zulkifly *et al.*, 2012). *Emticicia* sp. was found to slightly reduce the growth rate of axenic *Chlorella vulgaris* in co-cultivation, but the co-culture revealed prolonged stationary phase (Vu *et al.*, 2010).

For a number of strains (e.g. YP206, PAL10, PAL110, DMSP31), the observed effect of addition of the strain to liquid cultures of *Nannochloropsis* sp. CCAP211/78 (Fig. 1) was not in line with the trend observed for the same combination on solid agar (Fig. 2). This discrepancy between two screening methods corroborates that algae–bacteria interactions are complex and may vary under different culture conditions. Therefore, preliminary screening results should be confirmed by other methods such as flask cultures or bioreactors before claims regarding beneficial effects of bacteria on large-scale algal growth can be made.

Conclusion

In this study, we isolated 18 bacterial strains from two outdoor photobioreactors for cultivation of microalgae. A *Maritalea porphyrae* strain and a *Labrenzia aggregata* strain significantly promoted growth of *Nannochloropsis* sp. CCAP211/78 in liquid cultures in well plates (14% and 12% increase of the maximum chlorophyll concentration compared to the controls, respectively), and the *Labrenzia aggregata* strain also notably increased growth of the alga on agar plates. In addition, one strain most closely related to *Aquaticitalea lipolytica* significantly reduced the chlorophyll content with 28% compared to the axenic and non-axenic controls. Our results suggest that some bacteria from algal production systems may have pronounced impacts on algal growth under controlled laboratory conditions, an effect that should be verified for larger-scale algae cultures. Our results indicate that in the practice of improving the production of microalgae, the bacterial community in algal inocula should be considered. If harmful bacteria are present, the inoculum should be replaced by an inoculum where these bacteria are absent to increase the cultivation success. Perhaps even more interesting, beneficial bacterial strains may be supplemented as a new means to improve algal productivity and culture stability.

Experimental procedures

Algal cultivation

Nannochloropsis sp. CCAP211/78 cultures used for bacterial isolation were obtained from one horizontal and one vertical tubular photobioreactor at AlgaePARC, Wageningen. *Nannochloropsis* sp. CCAP 211/78 was cultivated in seawater (Eastern Scheldt, the Netherlands) enriched with a nutrient stock solution resulting in the following final concentrations (in mM); $NaNO_3$, 25; KH_2PO_4, 1.7; Na_2EDTA, 0.56; $Fe_2SO4{\cdot}7H_2O$, 0.11; $MnCl_2{\cdot}2H_2O$, 0.01; $ZnSO_4{\cdot}7H_2O$, $2.3{\bullet}10^{-3}$; $Co(NO_3)_2{\cdot}6H_2O$, $0.24{\bullet}10^{-3}$; $CuSO_4{\cdot}5H_2O$, $0.1{\bullet}10^{-3}$; $Na_2MoO_4{\cdot}2H_2O$, $1.1{\bullet}10^{-3}$. For the cultivation in outdoor photobioreactors, seawater was chemically sterilized by using sodium hypochlorite. Active hypochlorite was deactivated by filtration over active carbon, followed by filtration across a filter with a pore size of 1 μm. Subsequently, the nutrient stock solution was added through a sterile filter (0.45 μm). The detailed description of the cultivation process was given by de Vree *et al.* (2016).

For the co-cultivation experiment, the non-axenic precultures of *Nannochloropsis* sp. CCAP211/78 (100 ml liquid volume in 250 ml Erlenmeyer flasks) were maintained in autoclaved seawater supplemented with HEPES (20 mM) and Na_2EDTA (5 mM). The nutrient

stock solution with the same final concentrations as above was added to the autoclaved seawater through a syringe filter (0.2 μm). We refer to this medium as enriched seawater medium (ESW medium). The Erlenmeyer flask cultures of *Nannochloropsis* were capped with AeraSeal sterile film (Alphalabs, Eastleigh, UK) and placed in an orbital shaker incubator (Sanyo, Osaka, Japan), shaken at 120 rpm, illuminated with continuous light of 50 μmol photons m^{-2} s^{-1} at 25°C, and the headspace was enriched with 2 % CO_2.

Generation of axenic algal cultures

Axenic cultures of *Nannochloropsis* sp. CCAP211/78 were prepared using a treatment with an antibiotics cocktail consisting of Streptomycin (50 μg ml^{-1}), Gentamycin (67 μg ml^{-1}), Ciprofloxacin (20 μg ml^{-1}), Ampicillin (100 μg ml^{-1}) and Chloramphenicol (2.2 μg ml^{-1}). Specifically, 2 ml exponentially growing non-axenic *Nannochloropsis* sp. CCAP211/78 was taken from a 250 ml flask, washed twice in 2 ml sterile ESW medium and concentrated by centrifugation at 8000 g for 3 min and transferred into a six-well microplate. After adding the abovementioned antibiotics cocktail, the plate was incubated at 25 °C and illuminated with a 16/8 h light/dark cycle with a light intensity of 50 μmol photons m^{-2} s^{-1}. Every two days, the cultures in the well plate were washed as mentioned before, fresh antibiotics solution was added and the cultures were incubated as described above. This procedure was repeated four more times.

Axenicity of *Nannochloropsis* sp. CCAP211/78 was confirmed via inoculating ESW-YP agar (sterile ESW supplemented with 1 g l^{-1} of yeast extract, 1 g l^{-1} of peptone and 15 g l of agar) with 100 μl of antibiotics-treated algal cultures. Furthermore, algal cultures were incubated for 10 min with 1 μg ml^{-1} 4',6-diamidin-2-phenylindol (DAPI, dissolved in phosphate buffer). Stained samples were inspected with a fluorescence microscope (Olympus). The obtained axenic *Nannochloropsis* sp. culture was maintained in 250 ml flasks in the orbital shaker incubator (Sanyo) according to the same method as described for the non-axenic culture.

DNA isolation and 16S rRNA gene profiling of bacteria

Four samples of 5 ml from two outdoor photobioreactors were vacuum-filtered onto a cellulose nitrate membrane filter (0.2 μm; Millipore). To isolate the genomic DNA, filters were cut in half using sterile scissors and DNA was extracted from half a filter using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA) with the aid of a Precellys bead beater (Bertin Technologies Montigny-le-Bretonneux, France) with two rounds of bead beating for 45 s at speed of 5500 m s^{-1}.

Amplicons of the 16S rRNA gene were generated from the extracted DNA with a two-step PCR reaction carried out in a BIOKÉ SensoQuest Labcycler 48. During the first step of 16S rRNA gene PCR, a gene amplicon of approximately 311 bp was generated using degenerate primers 27F-DegS (Van den Bogert *et al*., 2011) and a mixture of 338R-I and 338R-II that comprise the V1 and V2 regions (Daims *et al*., 1999). The forward primer was used with Unitag1 attached to the 5' end of the primer and Unitag2 was attached to the 5' end of the reverse primer to facilitate the second step of the PCR (Table S3). The first PCR reaction (50 μl) contained 10 μl 5 × HF buffer (Thermo Scientific, the Netherlands), 1 μl dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 500 nM of Unitag1-27F-DegS forward primer, 500 nM of Unitag2-338R I and II reverse primer and 1 μl template DNA. The PCR was performed using the following conditions: an initial denaturation at 98°C for 30 s, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 20 s, elongation at 72°C for 20 s and a final extension at 72°C for 10 min. Subsequently, the first PCR product was used as template in a second PCR in order to add sample-specific barcodes (eight nucleotides). The second PCR reaction (100 μl) contained 20 μl 5 × HF buffer, 2 μl dNTP Mix, 2 U of Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 500 nM of a forward and reverse primer equivalent to the Unitag1 and Unitag2 sequences, respectively, that were each appended with an 8 nt sample-specific barcode (Table S3) (Tian *et al*., 2016). The second PCR was performed using the following conditions: an initial denaturation at 98°C for 30 s, followed by five cycles of denaturation at 98°C for 10 s, annealing at 52°C for 20 s, elongation at 72°C for 20 s and a final extension at 72°C for 10 min. The barcoded PCR products from the second PCR were purified using the High-Prep PCR purification kit (Magbio, London, UK) according to the manufacturer's protocols. The concentration of purified DNA was measured using a Qubit 2.0 Fluorometer, according to manufacturer's instructions for the Qubit dsDNA BR assay (Invitrogen, Grand Island, NY, USA). Then, the second step PCR products were pooled in an equimolar concentration and again purified using the High-Prep PCR purification Kit. The purified PCR products (final concentration: 200 ng μl^{-1}) were sequenced at GATC Biotech Europe (Konstanz, Germany, now part of Eurofins Genomics Germany GmbH) using the Illumina MiSeq Genome Sequencer platform. Nucleotide sequences of all samples were deposited at NCBI

GenBank under Bio Project ID number PRJNA488170 with accession number: SRR7760408.

Illumina sequencing data were processed and analysed using the NG-Tax pipeline (Ramiro-Garcia *et al.*, 2016) as previously described by (Dat *et al.*, 2018). Briefly, paired-end libraries were combined, and only read pairs with matching primers and barcodes were retained. Both forward and reverse reads were trimmed to 100 bp and concatenated to yield sequences of 200 bp that were used for subsequent sequence data processing. Demultiplexing, OTU picking, chimera removal and taxonomic assignment were performed within one single step. Reads were ranked per sample by abundance, and OTUs (at a 100% identity level) were added to an initial OTU table starting from the most abundant sequence until the abundance was lower than 0.1% per sample. The final OTU table was created by clustering the reads that were initially discarded (as they represented OTUs $< 0.1\%$ of the relative abundance) with the OTUs from the initial OTU table with a threshold of 98.5% similarity. Taxonomic assignment was done utilizing the UCLUST algorithm (Edgar, 2010) and the SILVA 111_SSU Ref database (Quast *et al.*, 2013; Yilmaz *et al.*, 2014).

Bacterial isolation and identification

Cryopreserved (15% glycerol) algal cultures from two outdoor reactors (three horizontal tubular bioreactor samples and one vertical tubular bioreactor sample) at AlgaeParc and stored at −80°C were used as inoculum for bacterial isolation. The description of bioreactors and algal cultivation processes was given by de Vree *et al.* (2016). Aliquots of cryopreserved cultures were diluted (10^4 fold) and plated on ESW agar (1.5% agar) supplemented with one of the following carbon sources: 2 g l^{-1} glucose (ESW-GLU); 2 g l^{-1} propionate (ESW-PRO); 2 g l^{-1} casamino acids (ESW-CAS); 2 g l^{-1} *Nannochloropsis* extract (ESW-ALG; 2 g freeze-dried *Nannochloropsis* cells from AlgaeParc suspended in 10 ml ESW, French-pressed two times at 110 MPa and centrifuged at 8000 g for 5 min. Subsequently, all the resulting supernatant was filter-sterilized (0.2 μm) and added to the autoclaved ESW medium of 1 l); 2 g l^{-1} succinate (ESW-SUC); 1 g l^{-1} yeast extract and 1 g l^{-1} peptone (ESW-YP); 2 g l^{-1} palmitate (ESW-PAL) or 0.6 mM dimethylsulphoniopropionate (ChemCruz, Dallas, TX, USA) (ESW-DMSP). Plates were maintained in the dark at room temperature. A fraction of the colonies (selection based on different colour and/or morphology) were picked and streaked until pure cultures were obtained. All the pure bacterial strains were maintained in ESW-YP medium. For bacterial identification, the 16S rRNA gene was amplified with universal primers 27F and 1492R (Table S3). The PCR reaction (50 μl) contained 10 μl 5 × HF buffer (Thermo Scientific, Waltham, MA, USA), 1 μl dNTP Mix (10 mM; Promega Leiden, The Netherlands)), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 1 μM of 27F primer, 1 μM of 1492R primer and 1 μl bacterial culture. The PCR protocol consisted of a pre-denaturation step (10 min at 98 °C) followed by 30 cycles of denaturation (30 s at 98°C), annealing (40 s at 60°C) and elongation steps (1.5 min at 72°C) with a final elongation step at 72°C for 10 min. The purified amplicons (High-Prep PCR purification kit, MOBIO) were Sanger sequenced with primer 806R (Table S3) by GATC Biotech Europe (Konstanz, Germany). The sequences were clustered into contigs with a cut-off of 99% similarity using ContigExpress (Invitrogen). One representative isolate from each contig was selected (Table 1) and analysed with the BLASTn tool from NCBI (http://www.ncbi.nlm.nih.gov/) to determine its phylogenetic affiliation. All sequences of selected isolates were deposited at NCBI GenBank under accession numbers as listed in Table 1. 16S rRNA gene sequences of the most closely related type strains were downloaded from NCBI GenBank. A phylogenetic tree was constructed with the FastTree online program (GTR model and Gamma model for likelihoods) (https://www.arb-silva.de/aligner/).

Co-culturing of algae with bacteria in microplates

For co-cultivation in 24-well microplates, both exponentially growing axenic and non-axenic cultures of *Nannochloropsis* sp. CCAP211/78 grown in flasks were diluted with ESW medium supplemented with 5 mM $NaHCO_3$ to a fluorescence intensity of ~ 5000 (Excitation: 450 nm, Emission: 685 nm). Single bacterial strains, separately grown in ESW-YP broth, were washed twice in sterile ESW medium and concentrated by centrifugation (8000 g for 5 min), then re-suspended in ESW medium and diluted to an OD600 of 0.2. Subsequently, 1 ml of axenic *Nannochloropsis* and 50 μL of bacterial strain suspension were inoculated in 24-well plates. The control cultures (either 1 ml of axenic *Nannochloropsis* sp. CCAP211/78 or 1 ml of non-axenic *Nannochloropsis* sp. CCAP211/78) were supplemented with 50 μL ESW medium instead of diluted bacteria. All treatments included three replicates and were randomly allocated into different wells of the microplate. The microplates were incubated in a custom-made LED box with one LED for each well and continuously illuminated at a light intensity of 71.1 ± 6.2 μmol photons m^{-2} s^{-1} (Fig. 3). The LED box was then placed in a shaking incubator (Innova, New Brunswick), agitated at 180 rpm min^{-1} and incubated at a temperature of 23 ± 1°C. Fluorescent intensity (Excitation: 450 nm, Emission: 685 nm) of co-cultures was measured at the same time every day with a plate reader in the endpoint

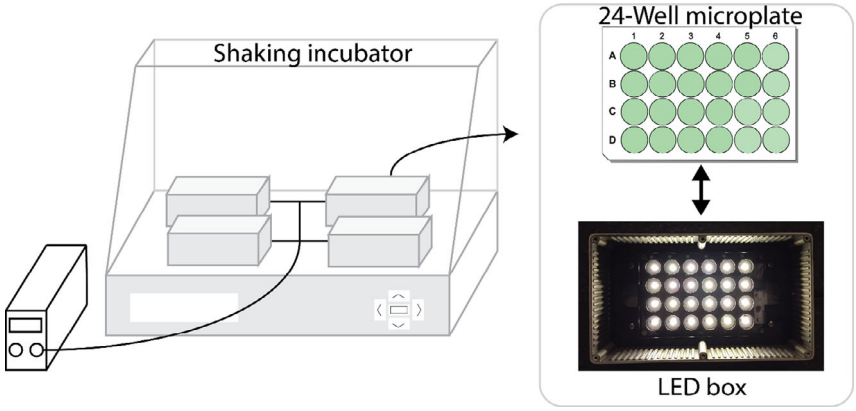

Fig. 3. Diagram illustrating the 24-well plate coupled with LED box for co-cultivation in a shaking incubator.

mode (BioTek Synergy, Winooski, VT, USA). Fluorescence intensity was measured from the bottom at 8 mm read height and 100 ms delays after plate movement. We determined that the relative fluorescent intensity was linearly correlated to cell counts of *Nannochloropsis* (Beckman-Coulter, Multisizer3, Fullerton, CA, USA) (Pearson's $r = 0.98$, $P < 0.0001$) (Fig. S1). Statistical test of comparison between treatments was done using a *t*-test and the *p* value was adjusted with the 'Holm' method (Holm, 1979).

Co-culturing of algae with bacteria on agar plates

For mixed cultivation of algae and bacteria on agar plates, an ESW plate (2% agar, diameter Petri dish 94 mm diameter) was overlaid with 5 ml 0.5% ESW agar (top agar) containing axenic *Nannochloropsis* cells (~10^5 per millilitre). After solidification of the top agar, 15 μl of bacterial culture was dropped onto the surface of the top agar. The plates were incubated for one week at a light intensity of 50 μmol photons m^{-2} s^{-1} with a 16:8 h light/dark cycle at 25°C.

Acknowledgements

Jie Lian would like to thank China Scholarship Council (Grant Number: 201406310023).

Conflict of interest

The authors declare that they have no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Linear correlation between the cell density of axenic *Nannochloropsis* sp. CCAP211/78 as determined by direct cell counting and the corresponding relative fluorescence. Samples shown here were taken from a pilot experiment that was carried out in a microplate under the same conditions as for the co-cultivation experiments.

Fig. S2. Relative abundance of bacteria (class level) in samples from different photobioreactors. HD and VD refer to horizontal tubular bioreactor and vertical tubular bioreactor, respectively. The number in the sample name refers to the sampling time point (day-month). The total number of bacterial 16S rRNA gene reads for each sample was HD0105 (16,033), HD1205 (132,458), HD2807 (9,672) and VD2807 (4,762), respectively.

Fig. S3. Co-culturing of *Nannochloropsis* sp. CCAP211/78 with individual bacterial strains. For each group, relative fluorescence was calculated as the mean of triplicate samples.

Fig. S4. Phylogenetic tree of 18 selected bacterial isolates and their closest bacterial type strains.

Table S1. Growth media from which sequenced bacterial isolates were obtained. Genus and species indicate best BLAST hit. Abbreviation: ALG-algal extract (n = 10); CAS-casamino acids (n = 14); DMSP-dimethylsulfoniopropionate (n = 9); GLU-glucose (n = 16); PAL-palmitate (n = 10); PRO-propionate (n = 25); SUC-succinate (n = 11) and YP-yeast extract plus peptone (n = 43).

Table S2. Composition of OTUs in four outdoor photobioreactor cultures of *Nannochloropsis*. The OTUs highlighted in green are identical to 16S rRNA gene sequences from bacterial isolates, the ones highlighted in yellow are the closest match (94%–98% identity) to the corresponding 16S rRNA gene sequence of bacterial isolates. OTUs with relative abundance of more than 5 % in a reactor sample are highlighted in red.

Table S3. Oligonucleotides used in this study.

