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Isolation, characterisation and phylogenetic diversity of culturable bacteria associated with marine microalgae from saline habitats of south India

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ABSTRACT: Cultivated microalgae are an essential source of nutrition to several farmed finfish, shellfish and many other commercially significant aquaculture species. Knowledge of microalgae-associated microhabitat is important for the development of a successful, pathogen-free hatchery rearing system. Therefore, an attempt was made to isolate (1), characterise (2) and determine the phylogenetic diversity of (3) bacteria associated with cultured microalgae, which are used as live feeds in many finfish and shellfish hatcheries. From 10 selected microalgal cultures, 34 bacterial isolates were obtained with total bacterial counts of 10^1 to 10^5 CFU ml^{-1}. Most notably, we checked the presence of *Vibrio* spp., the major aquaculture pathogen in all tested microalgae and their absence suggests the suitability of these microalgae for use in aquaculture systems. Phylogenetic analysis based on 16S rDNA sequencing revealed that the bacterial phylotypes associated with these microalgae were affiliated to *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteriia* classes. The genus *Marinobacter* (47%) was found to be the most predominant cultivable bacterium followed by *Alteromonas, Labrenzia*, *Oceanicaulis*, *Ponticoccus*, *Stappia* and *Rheinheimera*. Bacteria belonging to the genera *Gaetbulibacter* and *Maritalea* were also detected and, to the best of our knowledge, this is the first report of association of these bacterial groups with microalgae. The biochemical, enzymatic and antibacterial characteristics and tolerance to various abiotic stress factors of these bacterial isolates are also described in the present paper. Altogether, the present study gives an insight into the phycosphere of cultivated microalgae, which can be further explored for improving the productivity and reliability of indoor and outdoor microalgal culture systems.

KEY WORDS: Microalgal–bacterial interaction · Microalgae · Associated bacteria · 16S rDNA · Phylogeny

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INTRODUCTION

Bacteria and microalgae are 2 numerically dominant groups of microbes in the aquatic ecosystem (Flandez 2011). It has been realised that there is a close association between them under natural as well as in experimental conditions (Sapp et al. 2007, Krohn-Molt et al. 2013). The 'phycosphere' is a region where microalgae release many nutritional exudates; thus it is a favourable microenvironment for diverse subsets of bacteria (Sapp et al. 2007, Natrah et al. 2014). Bacteria in the phycosphere can either be free-living or directly attached to the phytoplankton surfaces (Grossart 1999). Moreover, when microbial partners come closer to the surface of the phytoplankton, various molecular mechanisms that

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promote bacterial attachment on the surface of their host are activated (Geng & Belas 2010). In algal habitats, these interactions may be either symbiotic, parasitic, commensal or competitive (Grossart & Simon 2007, Fuentes et al. 2016, Ramanan et al. 2016).

Bacteria are found as close associates of microalgae cultured under laboratory conditions, and may have a direct influence on algal growth and metabolism (Schwenk et al. 2014). Very little is known about the selection of specific types of bacteria by the phytoplankton (Jasti et al. 2005, Giroldo et al. 2007); however, this is probably influenced by the chemical microenvironment created by the host (Penesyan et al. 2010). For example, *Silicibacter* sp. isolated from dimethylsulfoniopropionate (DMSP)-producing *Pfiesteria piscicida* dinoflagellate cultures showed chemotactic response to DMSP and other dinoflagellate molecules (Miller et al. 2004). Conceição et al. (2010) reported that microalgal cultures harbour a broad spectrum of bacteria belonging to the groups *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Bacilli*. These bacterial partners normally enhance the algal growth by producing growth-stimulating factors such as vitamins, minerals and other essential nutrients and also play a role in the regeneration and remineralisation of organic compounds (Natrah et al. 2014). Thus, the associated bacteria that decompose extracellular products of microlagae participate in biogeochemical cycling and play an important part in the microbial loop (Jasti et al. 2005, Sapp et al. 2007, Natrah et al. 2014). In some cases, the production of algicidal compounds by certain bacteria ensures the environmental balance in nutrient cycle and energy flow.

Marine microalgae are widely used as larval feeds or feed additives in the rearing of aquatic animals. The productivity of a hatchery system mainly depends on the quality and quantity of these live feeds (Flandez 2011). Also, maintenance of a proper balance of diverse microflora associated with these live feeds is essential for a successful culture environment in commercial hatcheries (Schulze et al. 2006, Natrah et al. 2014). Sometimes the microalgae might stimulate pathogenic bacteria, especially *Vibrio* spp., which exert an overall negative effect on the aquaculture rearing system (Natrah et al. 2014). For example, Elston et al. (2008) reported that microalgal stock cultures were contaminated with *Vibrio* spp. with concentrations as high as 2.01×10^6 CFU ml^{-1} in a shellfish hatchery system. Hence, it is crucial to know the phytoplankton and their associated microenvironment in order to attain stable and reliable microalgae cultivation. Against this background, the present work aims to isolate, characterise and determine the phylogenetic diversity of culturable bacteria associated with 10 commercially important marine microalgae grown in laboratory conditions.

MATERIALS AND METHODS

Microalgae strain selection and culturing

Ten molecular and biochemically characterised (Preetha 2017) microalgal strains isolated during 2009 to 2015 were selected, based on their significance as aquaculture live feed, from the microalgae culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, south India) (Table 1). All isolates were maintained in f/2 seawater medium as monoalgal cultures in the algal germplasm as described by Preetha et al. (2012).

Isolation of associated bacteria

For the isolation of associated bacteria, 10 ml of well grown microalgae (~13 to 15 d old culture) was filtered through a 1.2 µm membrane filter (Pall). The filter cake obtained was rinsed with 0.85% sodium chloride (NaCl) to remove the free living bacteria. The filtered microalgae were suspended in 1 ml of 0.85% NaCl and vortexed. The mixture was serially diluted and plated on Zobell Marine Agar (ZMA) (Himedia) and thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia, India). The plates were incubated at 30°C for 24 to 96 h. After incubation, the total colony counts were taken and morphologically different colonies were selected and purified. The purified isolates obtained were preserved and maintained as glycerol stocks at −80°C for future use.

Identification and molecular phylogeny of bacteria

The total genomic DNA was extracted from all bacterial isolates using a phenol-chloroform enzymatic extraction method (Sambrook & Russell 2001). 16S rDNA amplification was carried out using universal primers NP1F (5′-GAG TTT GAT CCT GGC TCA-3′) and NP1R (5′-ACG GCT ACC TTG TTA CGA CTT-3′) (Pai et al. 2010) and the PCR conditions described in Nair et al. (2012). The amplified PCR products were purified (HiPura PCR

Table 1. Details of microalgae used for the study of associated bacteria. Strain codes for all isolates start with MBTDCMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, south India). Samples from an 'unknown' source were maintained as pure culture in live feed collection at CMFRI. ND: GenBank accession number (Acc No.) not determined

Strain code	Taxon	Algal type	Class	GenBank Acc No.	Source
S107	*Synechococcus* sp.	Blue-green algae	*Cyanophyceae*	KM087987	Unknown
S002	*Isochrysis galbana*	Golden-brown algae	*Prymnesiophyceae*	JF708124	Unknown
S072	*Chlorella* sp.	Green algae	*Treboxiophyceae*	JF708157	Marine Research Hatchery, CMFRI[a]
S082	*Tetraselmis* sp.	Green algae	*Prasinophyceae*	JF708168	Seawater, Poompuhar, Tamil Nadu
S135	*Dunaliella salina*	Green algae	*Chlorophyceae*	JF708161	Seawater, Calicut, Kerala
S078	*Nannochloropsis oceanica*	Heterokont algae	*Eustigmatophyceae*	JF708165	Unknown
S019	*Thalassiosira* sp.	Centric diatom	*Bacillariophyceae*	ND	Mangalavanam mangrove, Cochin, Kerala
S065	*Chaetoceros* sp.	Centric diatom	*Bacillariophyceae*	JF708154	Seawater, Njarakkal, Kerala
S043	*Navicula* sp.	Pennate diatom	*Bacillariophyceae*	JF708144	FortKochi ship channel, Cochin, Kerala
S092	*Nitzschia* sp.	Pennate diatom	*Bacillariophyceae*	ND	Hypersaline lake, Pulikat, Tamil Nadu

[a]Obtained as an invader in *Arthrospira platensis* marine open-tank culture

product purification kit, Himedia) and sequenced by the Sanger sequencing method. The 16S rDNA sequences of the isolates were compared with the sequences available in the EzTaxon database and identified up to generic level (Kim et al. 2012). Multiple alignment was done through the CLUS TALW algorithm (Thompson et al. 1994) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei 1987). Evolutionary analysis was conducted in MEGA6 and tree topologies were evaluated by bootstrap analysis of 1000 data sets (Tamura et al. 2013). The distances were computed using the Kimura 2 parameter method. The 16S rDNA sequence of one isolate Mab 04 (*Alteromonas* sp.) was not included in the analysis: as the sequencing reaction of this isolate repeatedly failed with NP1F, the primer NP1R was used to sequence the products. All the obtained sequences were submitted to the NCBI GenBank (Accession Nos. KR004791 to KR004798 and KR004801 to KR004826).

Characterisation of bacterial isolates

Biochemical characterisation

The bacterial isolates (10^6 to 10^8 CFU ml^{-1}) were characterised for physiological and biochemical properties using the potassium hydroxide (3% KOH) string test, oxidase, catalase, citrate utilisation, decarboxylation of arginine, ornithine and lysine and fermentation of different sugars following standard microbiological methods (Krieg & Holt 1984). The media used in this study were prepared with 1% sea salt (Sigma).

Enzymatic assay

All bacterial isolates were screened for the production of various hydrolytic exoenzymes such as amylase, casease, lipase, gelatinase, cellulase and urease (Nair et al. 2012). Purified bacterial isolates were spotted in the substrate amended nutrient agar prepared with 1% sea salt and were incubated at 30°C for 48 to 72 h. The activity was measured as the growth or zone around colonies with or without addition of reagents.

Antibacterial assay

The isolates were tested for antagonistic activity against 5 common aquaculture bacterial pathogens using a well diffusion assay (Valgas et al. 2007). The pathogens used were *Vibrio harveyi* 101, *V. anguillarum* A1, *V. alginolyticus* 101 (Central Institute of Brackishwater Aquaculture, Chennai), *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (Microbial Type Culture Collection, Chandigarh). Overnight grown cultures were inoculated into a freshly prepared Zobell marine broth (Himedia, India) and incubated for 48 h. After incubation, 50 µl of cell free suspension was poured into the wells punctured on the pathogen preswabbed Mueller Hinton Agar (Himedia) plates with 1% NaCl. These were then incubated at 30°C for 24 to 48 h and the results were recorded as a zone of inhibition observed around the wells.

Abiotic stress tolerance assay

The bacterial tolerance to fluctuating physiochemical conditions were detected by growth in gradients

of salinity, temperature and pH (Nair et al. 2012). To detect salinity tolerance, the isolates were inoculated into medium (0.5% peptone; 0.3% yeast extract; pH 7 ± 0.2) (Himedia) supplemented with different concentrations (0, 2, 5 and 10% w/v) of sea salt (Sigma). To study the temperature and pH tolerance, isolates were grown on ZMA plates incubated at 20 to 60°C and pH 5 to 9, respectively.

RESULTS AND DISCUSSION

It is now established that both culture-dependent and independent techniques for understanding the diversity of bacteria have their own bias problems and neither technique can simply be substituted for the other (Al-Awadhi et al. 2013). Our ultimate goal is to explore the growth-stimulating role of bacteria associated with microalgae for final application in aquaculture. For such studies we need cultivable microorganisms. In our study, 34 morphologically and biochemically different bacterial isolates (Strain Codes Mab 01 to Mab 36[01–22 and 25–36], see Table 2 below) were obtained from ZMA plates inoculated with suspensions of marine microalgal cultures. Their total counts in microalgal cultures were in the range of 10^1 to 10^5 CFU ml^{-1} and all the isolates obtained were Gram-negative. This was in agreement with the studies of Simidu et al. (1971) and Sini (2012) which reported the dominance of Gram-negative bacteria in the colonisation and association of microalgae. However, these findings contradict those of one previous study that reported that most of the isolates obtained from microalgal cultures of *Tetraselmis chuii* and *Chlorella minutissima* were Gram-positive (Makridis et al. 2006**)**. There was no bacterial growth on TCBS agar plates, which indicates the absence of the aquaculture pathogen *Vibrio* spp. in all of the selected microalgal cultures. Similar results are reported in several studies, even though this bacterial genus is ubiquitous in marine environments (Salvesen et al. 2000, Makridis et al. 2006, Conceição et al. 2010). The absence of *Vibrio* may be due to competitive exclusion by phycosphere bacteria, whereby they outcompete the pathogens and prevent their invasion of the niche which they already occupy (Natrah et al. 2014, Santos & Reis 2014). Thus, our findings indicate that it is safe to use these strains of microalgae as a live feed in aquaculture rearing systems.

Phylogenetic analysis of bacterial isolates based on partial 16S rDNA sequences showed they shared 94 to 100% identity with known bacterial genera. The 16S rDNA sequences of the isolates showed maximum similarity to the genera *Marinobacter*, *Alteromonas, Labrenzia, Ponticoccus, Oceanicaulis, Stappia, Gaetbulibacter, Maritalea* and *Rheinheimera*. Details of the isolates and their accession numbers are shown in Table 2. A neighbour-joining tree was constructed with 16S rDNA sequences, separated the isolates into 3 different clades as *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteriia* (Fig. 1). Out of the 34 culturable bacterial isolates obtained, 22 were from the class *Gammaproteobacteria*, distributed among genera such as *Marinobacter*, *Rheinheimera* and *Alteromonas.* From the class *Alphaproteobacteria*, 11 isolates belonged to 5 different genera *Labrenzia, Ponticoccus, Oceanicaulis, Maritalea* and *Stappia.* From the class *Flavobacteriia,* only 1 isolate, belonging to the genus *Gaetbulibacter*, was documented. Our results confirm earlier reports that microalgae were allied to these bacterial classes (Nicolas et al. 2004, Sapp et al. 2007, Conceição et al. 2010, Amin et al. 2012, Le Chevanton et al. 2013).

Mircoalgae, such as *Chaetoceros* sp., *Thalassiosira* sp. and *Nannochloropsis oceanica*, harbour diverse culturable bacterial groups belonging to 4 different genera. At the same time, only 1 genus, *Marinobacter*, was encountered in the phycosphere of *Nitzschia* sp., *Dunaliella salina* and *Chlorella* sp.. However, Schwenk et al. (2014) isolated *Loktanella* sp. and *Agrobacterium* sp. from *Chlorella pyrenoidosa* and *Nitzschia microcephala* cultures. Also, Guo & Tong (2014) isolated 3 symbiotic bacterial strains from *Chlorella vulgaris* ATCC 13482 culture, which were shown to be close relatives of *Pseudomonas alcaligenes*, *Elizabethkingia miricola* and *Methylobacterium radiotolerans*. We detected *Rheinheimera* sp. only from the diatom *Thalassiosira* sp. even though there is a report of its isolation from other diatom aggregates (Grossart et al. 2009). Similarly, Schwenk et al. (2014) reported the isolation of *Flexibacter* sp., *Seohaeicola saemankumensis*, *Roseobacter* sp. and *Erythromicrobium* sp. from laboratory-maintained cultures of *Isochrysis* sp. However, we obtained bacterial isolates belonging to the genera *Labrenzia* and *Alteromonas* from *Isochrysis galbana* culture. At the same time, the study by Sharifah & Eguchi (2011) supports our isolation of *Stappia* sp. from *Nannochloropsis oceanica* culture. Concurrently, we isolated the bacterial genera *Maritalea* and *Gaetbulibacter* from the diatom *Navicula* sp. The bacterial genus *Marinobacter* was isolated from most of the microalgal species (*Synechococcus* sp., *Chlorella* sp., *Dunaliella salina, Nannochloropsis oceanica, Chaetoceros* sp., *Navicula* sp. and *Nitzschia* sp.), and it comprised 47%

Table 2. Identification of culturable bacteria associated with microalgae using 16S rDNA sequence data. Strain code for all bacterial isolates starts with MBTDCMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, south India)

Source microalga (taxon)	Isolated bacterial strains: Strain code	GenBank accession no.	Bacterial count (CFU ml-1)	Phylogenetic group	Reference strains in EzTaxon database: Closest relatives	Similarity (%)	GenBank accession no.
Chaetoceros sp.	Mab 01	KR004810	4.6×10^4	*Marinobacter* sp.	*Marinobacter salsuginis*	99.93	EF028328
	Mab 02	KR004811	3.8×10^4	*Oceanicaulis* sp.	*Oceanicaulis stylophorae*	98.84	HM035090
	Mab 03	KR004812	2.3×10^4	*Labrenzia* sp.	*Labrenzia suaedae*	99.56	GU322907
	Mab 04	KR004826	2×10^2	*Alteromonas* sp.	*Alteromonas marina*	99.42	AF529060
Thalassiosira sp.	Mab 05	KR004813	2.35×10^5	*Oceanicaulis* sp.	*Oceanicaulis stylophorae*	99.39	HM035090
	Mab 06	KR004814	3×10^2	*Ponticoccus* sp.	*Ponticoccus litoralis*	96.64	EF211829
	Mab 07	KR004824	1×10^2	*Alteromonas* sp.	*Alteromonas macleodii*	99.33	CP003841
	Mab 08	KR004815	4×10^1	*Rheinheimera* sp.	*Rheinheimera aquimaris*	99.00	EF076757
Nitzschia sp.	Mab 09	KR004816	9.8×10^3	*Marinobacter* sp.	*Marinobacter salsuginis*	99.71	EF028328
	Mab 10	KR004817	1.02×10^5	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	100.00	FO203363
	Mab 11	KR004818	3.8×10^4	*Marinobacter* sp.	*Marinobacter salsuginis*	99.71	EF028328
Navicula sp.	Mab 12	KR004819	8×10^3	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	100.00	FO203363
	Mab 13	KR004820	9.6×10^2	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	98.54	FO203363
	Mab 14	KR004821	1×10^3	*Maritalea* sp.	*Maritalea mobilis*	100.00	EU255260
	Mab 15	KR004791	2×10^3	*Gaetbulibacter* sp.	*Gaetbulibacter jejuensis*	100.00	FJ490367
D. salina	Mab 16	KR004792	1.96×10^4	*Marinobacter* sp.	*Marinobacter salsuginis*	99.93	EF028328
	Mab 17	KR004793	2×10^4	*Marinobacter* sp.	*Marinobacter algicola*	98.08	ABCP01000031
	Mab 18	KR004794	1.2×10^4	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	99.71	FO203363
Chlorella sp.	Mab 19	KR004795	5.1×10^3	*Marinobacter* sp.	*Marinobacter vinifirmus*	98.15	DQ235263
	Mab 20	KR004796	6.4×10^3	*Marinobacter* sp.	*Marinobacter vinifirmus*	98.00	DQ235263
Tetraselmis sp.	Mab 21	KR004797	4.47×10^5	*Labrenzia* sp.	*Labrenzia aggregata*	100.00	AAUW01000037
	Mab 22	KR004798	3×10^1	*Alteromonas* sp.	*Alteromonas macleodii*	99.42	CP003841
I. galbana	Mab 25	KR004801	2.5×10^3	*Alteromonas* sp.	*Alteromonas macleodii*	99.50	CP003841
	Mab 26	KR004822	1×10^3	*Labrenzia* sp.	*Labrenzia aggregata*	100.00	AAUW01000037
N. oceanica	Mab 27	KR004823	2×10^3	*Marinobacter* sp.	*Marinobacter algicola*	94.17	ABCP01000031
	Mab 28	KR004802	1×10^3	*Labrenzia* sp.	*Labrenzia aggregate*	100.00	AAUW01000037
	Mab 29	KR004803	1.4×10^4	*Stappia* sp.	*Stappia stellulata*	99.85	AUIM01000013
	Mab 30	KR004804	6×10^3	*Marinobacter* sp.	*Marinobacter lipolyticus*	99.29	ASAD01000031
	Mab 31	KR004825	4×10^3	*Alteromonas* sp.	*Alteromonas macleodii*	99.28	CP003841
Synechococcus sp.	Mab 32	KR004805	5×10^2	*Marinobacter* sp.	*Marinobacter vinifirmus*	97.97	DQ235263
	Mab 33	KR004806	5×10^2	*Stappia*sp.	*Stappia stellulata*	99.85	AUIM01000013
	Mab 34	KR004807	9.8×10^4	*Marinobacter* sp.	*Marinobacter vinifirmus*	98.00	DQ235263
	Mab 35	KR004808	6.4×10^4	*Ponticoccus* sp.	*Ponticoccus litoralis*	98.40	EF211829
	Mab 36	KR004809	2.4×10^4	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	99.71	FO203363

of total bacterial isolates obtained. Their predominance indicates their close affiliation with the phycosphere of diverse groups of microalgae (Jasti et al. 2005, Amin et al. 2012, Natrah et al. 2014). Likewise, many previous studies report a microalgal association with *Alteromonas*, which supports our findings on isolation of similar bacterial genera from *Nannochloropsis oceanica*, *Isochrysis galbana*, *Tetraselmis* sp., *Thalassiosira* sp. and *Chaetoceros* sp. (Jasti et al. 2005, Sapp et al. 2007, Ali et al. 2010, Amin et al. 2012, Le Chevanton et al. 2013). Two other genera isolated in our study, *Oceanicaulis* and *Labrenzia*, were previously reported to be associated with the toxic dinoflagellates *Alexandrium tamarense* and *A. lusitanium*, respectively (Strömpl et al. 2003, Fiebig et al. 2013). Many culturable bacterial genera identified from our study were also previously documented to be associated with macroalgae; for example, *Marinobacter* and *Labrenzia* were associated with the green alga *Bryopsis* (Hollants et al. 2011, Hollants 2012), *Alteromonas* with seaweeds from the Gulf of Mannar (Janakidevi et al. 2013), *Stappia* with green alga *Ulva intestinalis* (Ali et al. 2010) and *Maritalea* with the red alga *Porphyra yezoensis* (Fukui et al. 2012). However, to the best of our knowledge, the isolate belonging to the genus *Gaetbulibacter* is reported here for the first time in an algal association.

The physiological and biochemical characteristics of the bacterial isolates are shown in Table 3. All the isolates were oxidase positive except Mab 03 (*Labrenzia* sp.), Mab 20 and Mab 34 (*Marinobacter* spp.). Only 6 bacterial isolates Mab 02 (*Oceanicaulis*

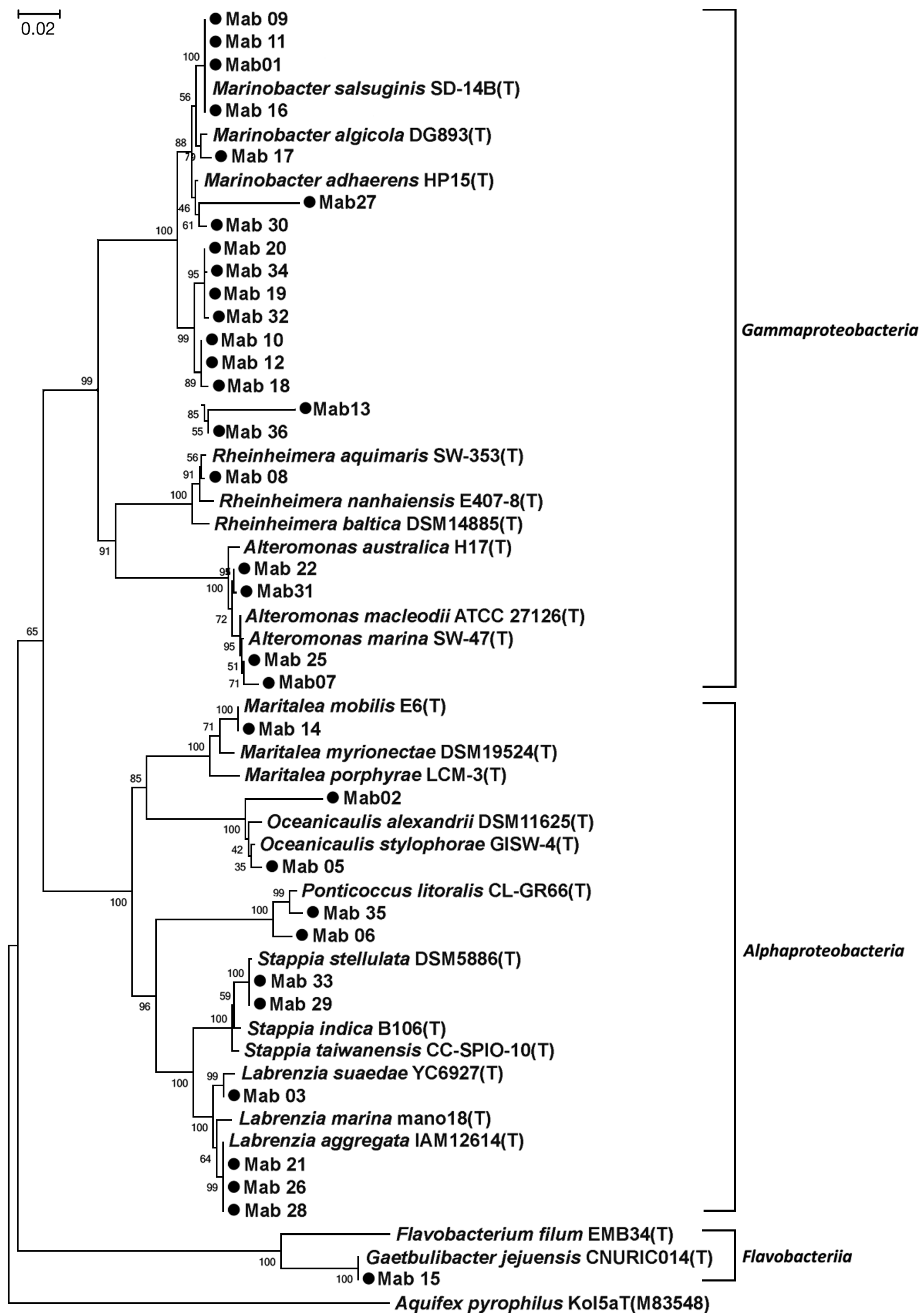

Fig. 1. Neighbour-joining phylogenetic tree based on partial 16S rDNA sequence of culturable bacterial strains isolated by this study (strain codes Mab 01 to Mab 36) and reference strains from the EzTaxon database (see Table 2)

Table 3. Physiological and biochemical characteristics (positive or negative) of culturable bacterial isolates associated with microalgae (see Table 2). A: KOH string test; B: oxidase; C: catalase; D: arginine; E: ornithine; F: lysine; G: citrate; H: sucrose; I: sorbitol; J: lactose; K: maltose; L: mannose; M: arabinose; N: galactose; O: xylose; P: glucose; Q: raffinose

Strain	Biochemical tests			Decarboxylation			Utilisation	Fermentation									
code	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Mab 01	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 02	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 03	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Mab 04	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 05	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 06	+	+	+	−	+	−	+	−	−	−	−	+	−	−	−	−	−
Mab 07	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 08	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 09	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 10	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 11	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 12	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 13	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 14	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 15	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 16	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 17	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Mab 18	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 19	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 20	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 21	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+	−	−
Mab 22	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 25	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 26	+	+	+	−	+	−	+	−	−	−	−	−	−	−	+	−	−
Mab 27	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 28	+	+	+	−	+	−	+	−	−	−	−	−	−	−	+	−	−
Mab 29	+	+	+	−	+	−	+	−	−	−	+	−	−	−	−	−	−
Mab 30	+	+	−	−	+	−	+	+	−	+	+	+	−	+	+	+	+
Mab 31	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 32	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 33	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Mab 34	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Mab 35	+	+	+	+	+	−	+	−	−	−	+	+	−	−	−	+	−
Mab 36	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−

sp.), Mab 09, Mab 10, Mab 13, Mab 18 and Mab 30 (all *Marinobacter* spp.) were catalase negative. In the decarboxylation assay, most of the isolates were positive for at least 1 substrate, except the cultures Mab 03 (*Labrenzia* sp.) and Mab17 (*Marinobacter* sp.) which were negative in all the 3 tests. The metabolic utilisation of tested sugars was lacking in most of the isolates, since they were found to be fermentation negative. However, the isolates Mab 30 (*Marinobacter* sp.) and Mab 35 (*Ponticoccus* sp.) were able to ferment many sugars (sucrose, lactose, maltose, mannose, galactose, xylose, glucose and raffinose) as shown in Table 3. Also all *Labrenzia* spp. (Mab 03, Mab 21, Mab 26 and Mab 28) obtained from our study were xylose fermenters. Brown (1991) reported differences in the sugar composition of polysaccharides from microlagae belonging to different species and classes. This variation in the sugar composition might contribute to the difference in the sugar fermentation capability of their bacterial counterparts.

Many culturable bacterial isolates associated with microalgal cultures were capable of producing the hydrolytic exoenzymes gelatinase (61.8%) and urease (58.8%). Only 44.1 and 35.3% of the isolates exhibited lipase and amylase activity, respectively. Mab 33 (*Stappia* sp.) associated with *Synechococcus* sp. was the only isolate detected positive for cellulase, and none of the isolates exhibited casease activity (Table 4). Our results indicate that most of the isolated bacterial strains can hydrolyse algal exudates and act as remineralisers of various organic compounds which leads to the release of nitrogen, phosphorus and carbon compounds to the phycosphere. This can enhance and sustain the growth of micro-

Table 4. Enzymatic and abiotic stress tolerance assay of culturable bacterial isolates associated with microalgae (see Table 2). Results of enzymatic assays are shown as (–) no enzymatic activity, (+) zone in diameter 10 to 20 mm or (++) zone in diameter 20 to 30 mm. R: lipase; S: amylase; T: cellulase; U: casease; V: urease; W: gelatinase. Results of tolerance assays are shown as ranges. X: hydrogen ion concentration (pH); Y: Temperature (°C); Z: Salinity (ppt)

Strain	Enzymatic assay						Tolerance assay		
code	R	S	T	U	V	W	X	Y	Z
Mab 01	+	–	–	–	+	–	5–9	20–45	20–100
Mab 02	–	–	–	–	++	+	5–9	20–45	20–100
Mab 03	–	–	–	–	++	–	5–9	20–45	20–50
Mab 04	+	+	–	–	–	++	5–9	20–45	20–100
Mab 05	–	–	–	–	–	+	5–9	20–45	20–100
Mab 06	–	–	–	–	–	+	6–9	20–45	20–50
Mab 07	+	+	–	–	–	++	5–9	20–45	20–100
Mab 08	+	++	–	–	++	++	5–9	20–45	20–100
Mab 09	–	–	–	–	–	+	5–9	20–45	20–100
Mab 10	+	–	–	–	+	+	5–9	20–45	20–100
Mab 11	+	–	–	–	+	–	5–9	20–45	20–100
Mab 12	+	–	–	–	+	+	5–9	20–45	20–100
Mab 13	+	–	–	–	+	+	5–9	20–45	20–100
Mab 14	–	–	–	–	–	+	6–9	20–45	20–100
Mab 15	–	+	–	–	–	+	5–8	20–40	20–100
Mab 16	+	–	–	–	–	–	5–9	20–45	20–100
Mab 17	–	++	–	–	–	–	5–9	20–40	20–100
Mab 18	+	–	–	–	+	+	5–9	20–45	20–100
Mab 19	–	++	–	–	+	+	5–9	20–45	20–100
Mab 20	–	++	–	–	–	+	6–9	20–45	20–100
Mab 21	–	–	–	–	++	–	5–9	20–45	20–100
Mab 22	+	+	–	–	–	++	5–9	20–45	20–100
Mab 25	+	+	–	–	–	++	5–9	20–45	20–100
Mab 26	–	–	–	–	++	–	5–9	20–45	20–100
Mab 27	+	–	–	–	+	–	5–9	20–45	20–100
Mab 28	–	–	–	–	++	–	5–9	20–45	20–100
Mab 29	–	–	–	–	++	+	5–9	20–45	20–100
Mab 30	–	–	–	–	–	–	5–9	20–45	20–100
Mab 31	+	+	–	–	–	++	5–9	20–45	20–100
Mab 32	–	++	–	–	+	+	6–9	20–45	20–100
Mab 33	–	–	+	–	++	–	5–9	20–45	20–100
Mab 34	–	++	–	–	+	+	5–9	20–45	20–100
Mab 35	–	–	–	–	++	–	5–9	20–40	20–50
Mab 36	+	–	–	–	++	–	5–9	20–45	20–100

algae for a prolonged period of time (Grossart 1999, Sini 2012, Natrah et al. 2014). The extracellular enzyme activity of microalgal cultures can also not be denied (Patil & Mahajan 2016). In nonaxenic microalgal cultures, it may be enhanced by the hydrolytic enzyme activity of bacteria associated with them.

We screened all the isolates for their antibacterial activity against 5 major aquaculture pathogens belonging to the genus *Vibrio*, but none of the isolates was found to possess antagonistic activity. The bacterial isolates were assessed for their tolerance against different abiotic stress factors like salinity, temperature and pH and the results are given in Table 4. In salinity tests, Mab 03 (*Labrenzia* sp.), Mab 06 and Mab 35 (*Ponticoccus* spp.) showed growth only up to 50 ppt. The rest of the isolates showed growth up to 100 ppt indicating that most of them are halotolerant species. All the isolates grew well at 20°C. The maximum temperature tolerance, beyond which no growth was observed, for Mab 15 (*Gaetbulibacter* sp.), Mab 17 (*Marinobacter* sp.) and Mab 35 (*Ponticoccus* sp.) was 40°C. All other isolates showed growth up to 45°C. Mab 06 (*Ponticoccus* sp.), Mab 14 (*Maritalea* sp.), Mab 20 and Mab 32 (*Marinobacter* spp.) showed maximum growth between pH 6 and 9, whereas Mab 15 (*Gaetbulibacter* sp.) showed optimum growth at pH 5 to 8. All other isolates grew well at pH 5 to 9. Thus, our results showed that microalgae cultures are associated with versatile groups of bacteria that can survive under diverse physiological stresses. Open mass culture of marine microalgae takes place in conditions marked by seasonal fluctuations in salinity, temperature etc. (Adenan et al. 2013). Hence, the abiotic stress tolerance would help better adaptation of these bacterial strains towards their phytoplankton host.

The results of the present study clearly indicate the existence of a strong and close association between bacteria and microalgae, including under artificial conditions. which makes the phycosphere a hotspot of complex interactions (Sapp et al. 2007, Schwenk et al. 2014). When compared to algae, associated bacterial biomass is low but it can complement the live feed used in aquaculture with many growth factors and improve success rates in larval rearing (Nicolas et al. 2004). However, in addition to beneficial effects, many associated bacterial groups are reported to display algicidal activity (Natrah et al. 2014). Thus, the presence of bacteria plays a pivotal role in energy, nutrient and ecological balance (Cole 1982). Hence, in order to optimally benefit from microalgal–bacterial interaction, it is crucial to increase understanding of the various aspects of interactions which still remain unexplored. Current knowledge on bacterial groups associated with diverse microalgal hosts can be further extended to develop a consortium of suitable bacteria with wide applications in microalgal mass culture. Thus, the present study on microalgal bacterial flora will provide a basis for further research to improve stability, productivity and sustainability of large scale production of microalgae.

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