J Appl Phycol (2012) 24:525–534
DOI 10.1007/s10811-011-9758-0

Jania rubens-associated bacteria: molecular identification and antimicrobial activity

A. Ismail-Ben Ali · M. El Bour · L. Ktari · H. Bolhuis · M. Ahmed · A. Boudabbous · L. J. Stal

Received: 31 July 2011 / Revised and accepted: 31 July 2011 / Published online: 29 December 2011

Abstract Marine macroalgae surfaces constitute suitable substrata for bacterial colonization which are known to produce bioactive compounds. Thus, hereby we focused on heterotrophic aerobic bacteria species associated with coralline red alga *Jania rubens* (northern coast of Tunisia, southern Mediterranean Sea) and their inhibition against several microbial marine and terrestrial species. The whole collection (19 isolates, J1 to J19) was identified, based on their 16S ribosomal RNA gene sequences as Proteobacteria (14 strains), Bacteroidetes (4 strains) and Firmicutes (1 strain). Thirty-six percent of the isolates (J2, J9, J11, J13, J16, J17 and J18) were antibiotic-like producers with in vitro inhibition against Gram + and Gram − bacteria and the yeast *Candida albicans*. Highest level of inhibition was revealed for the isolates J2, J9 and J13 identified respectively as *Bacillus*, *Aquimarina* and *Pseudomonas*, with strong activity against *Staphylococcus aureus*, *Micrococcus* and *C. albicans*, with inhibition diameters of 25 to 35 mm shown by drop test assay on T soy agar plates. Furthermore, we tested inhibition of *J. rubens* crude organic extracts against human and marine bacteria as well as against all *J. rubens* isolates, in order to determine the degree of affinity of the epibionts to their proper host. The recovery of strains with antimicrobial activity suggests that *J. rubens* represent an ecological niche which harbors a specific microbial diversity worthy of further secondary metabolites investigation.

Keywords Antibacterial and antifungal activities · Bacteria · Seaweed epibionts · Rhodophyta

A. Ismail-Ben Ali · M. El Bour · L. Ktari
National Institute of Marine Sciences and Technologies (INSTM), Salammbô, Tunisia

A. Boudabbous
Faculty of Mathematical, Physical and Natural Sciences of Tunis, University of Tunis El Manar, Tunis, Tunisia

H. Bolhuis · M. Ahmed · L. J. Stal
Department of Marine Microbiology, Netherlands Institute of Ecology, NIOO-KNAW, PO Box 140, 4400 AC, Yerseke, The Netherlands

M. El Bour (✉)
Laboratory of Biotechnology and Marine Biodiversity, INSTM Salammbô, 28, Rue du 2 mars 1934, 2025, Salammbô, Tunisia
e-mail: monia.elbour@instm.rnrt.tn

Introduction

The increasing demand for new therapeutic drugs from natural products led to greater interest towards marine microorganisms which are prolific producers of bioactive secondary metabolites in response to ecological parameters such as competition for space and maintenance of un-fouled surfaces (Konig et al. 1994). Colonization of sessile eukaryotic host surfaces by bacteria is common in the marine environment (Thomas et al. 2008), and macroalgae have long been known to support abundant populations of bacteria (Armstrong et al. 2000; Rao et al. 2005). Seaweeds are highly productive components of the coastal ecosystem releasing dissolved organic carbon into surrounding waters thus harboring suitable living substrata for microbial colonization (Hengst et al. 2010). Chemically driven interactions are important in the establishment of cross relationships between marine surface-associated microorganisms and their eukaryotic host (Imhoff et al. 2011). Epiphytic bacteria produce antimicrobial compounds that may be protective for algae from colonization by other microbiota and macrobiota (Holmstrom et al.

2002; Rao et al. 2006, 2007; Hengst et al. 2010). Other benefits can be attributed to the symbiotic relation; Croft et al. (2005) suggested that seaweeds, as microalgae, may acquire vitamin B12 from closely associated bacteria.

Few data is available on epiphytic bacteria of red algae. Recently, Kanagasabhapathy et al. (2008) identified epibiotic bacteria of nine non-calcareous species of red algae from Japanese waters with antimicrobial activities. Other studies concerned the bacterial biota on crustose coralline algae (Lewis et al. 1985; Johnson et al. 1991; Barott et al. 2011). It has been suggested that the characteristic bacteria associated with such species influence invertebrate larvae recruitments. Sakami et al. (1999) studied epiphytic bacteria of the branched coralline alga *Jania* sp. and their effects on the growth of toxic dinoflagellate. Among the isolated bacteria, one *Flavobacterium* strain inhibited positively *Gambierdiscus toxicus*, a microalga known to be causative of ciguatera fish food poisoning. In addition, Boyd et al. (1999) reported antifouling properties of epiphytic bacteria associated with the calcareous red alga *Corallina officinalis*.

Despite the limitations of cultivation-based studies, cultivation remains essential as it provides opportunities to study and understand microbial ecology and physiology and design antibiotic screening assays (Mearns-Spragg et al. 1998; Armstrong et al. 2000).

The branched coralline alga *Jania rubens* is widespread along Tunisian coasts, especially during spring season. It has been studied for its cytotoxic activity (Ktari et al. 2000). Still, no data are available concerning its epibionts and their biological potential. Thus, the present study provides information on the diversity of culturable bacterial communities intimately associated with *J. rubens* surfaces as well as their antimicrobial potential against several human and fish pathogens.

Materials and methods

Samples of *J. rubens* (Linnaeus) J.V. Lamouroux were collected within the period of February to August 2007 from Cap Zebib zone (37° 16.2′ N, 10° 3.6′ E), northern coast of Tunisia. Algae samples were transferred in the dark in sterile plastic bags filled with water from same location.

Isolation of epiphytic bacteria

Seaweed samples were washed three times with autoclaved seawater to remove free living and associated bacteria (Gil-Turnes et al. 1989; Cheng et al. 1999; Jiang et al. 1999; Burgess et al. 2003). Subsequently, firmly attached epiphytic bacteria were extracted by vortexing 10 g of alga in 90 mL autoclaved seawater for 6 min. Bacteria were isolated by serial dilution to 10^{-3} using sterile seawater. From each dilution 100 μL was spread-plated in triplicate on marine agar (MA: Pronadisa Laboratories, CONDA). The plates were incubated at 20°C until colonies appeared or at least 7 days (Lemos et al. 1985). Visually distinct bacterial colonies were selected and further plated on MA until clonal cultures were obtained. The pure cultures were stored at −80°C in marine broth (Pronadisa Laboratories, CONDA) supplemented with 20% glycerol.

Extraction of DNA and PCR

Single colonies from plates were suspended in sterile MilliQ water and used as template in the PCR reactions using the universal 16S ribosomal RNA (rRNA) gene primers B8F and U1492R (Table 1). PCR reactions were performed using a Gene Biometra T1 DNA thermal cycler (Perkin-Elmer Co., USA) in 25 μL (final volume) reaction mixtures containing 0.1 μL of Hot start DNA polymerase (Sigma), each primer at a final concentration of 10 pmol μL^{-1}, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.25 μL of 100% DMSO (dimethylsulfoxide), 2.5 μL of BSA (bovine serum albumin) at 0.2 mg mL^{-1} final concentration, 2.5 μL of PCR buffer (including $MgCl_2$) and 1 μL of DNA template. The PCR protocol consisted of 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 50 s. The cycles were preceded by 15 min of denaturation at 94°C and ended with a final extension for 7 min at 72°C. Negative controls contained all of the components of the PCR mixture except the DNA template.

Analysis of PCR products

PCR products (2 μL) were analyzed by agarose gel electrophoresis (1% w/v agarose, 1× TAE running buffer containing 40 mM Tris acetate and 1 mM EDTA, pH 8). Electrophoresis was performed at 100 V for 45 min. The gels were stained for 45 min with SYBR Gold nucleic acid gel stain (Invitrogen Corp.) and photographed under UV illumination.

Table 1 Primers used for PCR and DNA sequencing

Primer	Sequence
B8F	5′-AGAGTTTGATCMTGGCTCAG-3′
U1492R	5′-GGTTACCTTGTTACGACTT-3′
C5	5′-AGAGTTTGATCCTGGCTCAGG-3′
C26	5′-GGGCGGTGTGTACAAGG-3′
C72	5′-CCGGAATIATTGGGCGTAA-3′
C112	5′-CTCGTTGCGGGACTTAACCC-3′

DNA sequencing

Approximately 100 ng template DNA was sequenced using the BigDye Terminator chemistry (Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystem) according to the manufacturer's instructions. The sequence products were analyzed on an ABI 377 DNA sequencing system (Applied Biosystem) using the primers C5, C26, C72 and C112 (Table 1).

Phylogenetic analysis

The closest phylogenetic relatives of each isolate were identified by comparison of the 16S rRNA gene sequence to the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis tools (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis of *J. rubens* isolates sequences and that of other red alga isolates sequences was performed using the Neighbor Joining method available in MEGA4.0 (Tamura et al. 2004).

Nucleotide sequence accession numbers

Nucleotide sequences of the isolates sequenced in this study have been added to the DNA GenBank with the following accession numbers: J1 (JN391160), J2 (JN391161), J3 (JN391162), J4 (JN391163), J5 (JN391164), J6 (JN391165), J7 (JN391166), J8 (JN391167), J9 (JN391168), J10 (JN391169), J11 (JN391170), J12 (JN391171), J13 (JN391172), J14 (JN391173), J15 (JN391174), J16 (JN391175), J17 (JN391176), J18 (JN391177) and J19 (JN391178).

Preparing algal extracts

About 20 g of dried and powdered alga was extracted consecutively with two organic solvents with increasing polarity: dichloromethane (D) and dichloromethane/methanol (D/M) (1:1 v/v). Each extraction was carried out three times by maceration for 24 h at room temperature. These extracts were pooled, filtered and concentrated under reduced pressure in a rotary evaporator apparatus. Extracts were stored at −20°C until use.

Antimicrobial activity

Antimicrobial activity of both algal isolates and algal extracts was evaluated against the following pathogen strains: *Escherichia coli* O126B16, *E. coli* ATCC 25922, *E. coli* ATCC 8739, *Vibrio tapetis* CECT4600, *V. anguillarum, V. alginoliticus* ATCC 17749 T, *Pseudomonas cepacia*, *P. fluorescens* AH2, *P. aeruginosa* ATCC 27853, *Aeromonas salmonicida*, *A. hydrophila*, *Salmonella typhimurium*, *Streptococcus* sp., *Staphylococcus aureus*, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *Enterococcus feacalis* ATCC 29212, *Micrococcus* and the yeast *Candida albicans*. Algal extracts were also tested against *Jania* isolates and four strains from alga surrounding water.

Bacterial isolates were screened for their antimicrobial activity against pathogens using drop test assay on T soy agar (TSA, BIO RAD) plates containing 20 g L^{-1} NaCl as described by James et al. (1996) and Rao et al. (2005) with slight modifications. Briefly, drops of the full cell suspension of an overnight culture were spotted onto the agar plates containing a confluent lawn of the target strain (dried at 30°C for 30 min) and subsequently incubated at 30°C. Growth inhibition was evaluated after incubation (24 h at 30°C) by measuring the zone of inhibition around the spots.

The antibacterial assay of *J. rubens* extracts against its proper isolates, indicators bacteria and four *Jania* surrounding water isolates was evaluated by using standard paper disc method. Briefly, 500 μg of the crude extract dissolved in appropriate solvent (10 μL) was applied to sterile filter paper discs (6 mm). After solvent evaporation, the discs were placed on TSA plates, inoculated with an 18-h cultured associated strain (10^6 bacteria mL^{-1}) in tryptic soy broth (TSB, BIO RAD) containing 20 g L^{-1} NaCl. As control, a disc loaded with solvent was simultaneously prepared. Plates were incubated overnight at 30°C. The diameter (in millimeters) of growth inhibition halo was measured after 24-h incubation. Assays were carried out in triplicate.

Results

In this study we first examined the composition on the culturable surface-associated bacterial community on *J. rubens* using 16S rRNA gene sequence analysis.

Nineteen epiphytic bacteria (J1–J19) were isolated as pure culture on marine agar. The 16S rRNA gene sequences revealed that the main bacterial groups present in the surface-associated community were Alphaproteobacteria and Gammaproteobacteria, Bacteroidetes and Firmicutes (Table 2). Results demonstrated that Proteobacteria (about 73%) constitute the majority of bacterial cells on the surface of *J. rubens* and were assigned especially to seven families, with the predominant one being *Rhodobacteraceae* containing five strains (J1, J3, J4, J7 and J16) followed by *Pseudomonadaceae* (J11, J13 and J14), *Pseudoalteromonadaceae* (J17 and J18), *Vibrionaceae* (J19), *Oceanospirillaceae* (J10) and

Table 2 Identification of 19 bacterial isolates associated with *J. rubens* based on partial 16S rRNA gene sequence identity obtained by BLAST analysis

Isolate	Number (bp)	Accession number	Closest match in GenBank	Identity (%)	*E* value	Phylum
J1	1132	JN391160	*Roseovarius aestuarii*	98	0.0	Alphaproteobacteria
J2	1198	JN391161	*Bacillus pumilus*	99	0.0	Firmicutes
J3	618	JN391162	*Paracoccus* sp.	97	0.0	Alphaproteobacteria
J4	772	JN391163	*Loktanella* sp.	99	0.0	Alphaproteobacteria
J5	1209	JN391164	*Shewanella sairae*	98	0.0	Gammaproteobacteria
J6	420	JN391165	*Flavobacteria bacterium*	100	0.0	Bacteroidetes
J7	1170	JN391166	*Rhodobacteraceae bacterium*	99	0.0	Alphaproteobacteria
J8	1178	JN391167	*Agrobacterium* sp.	99	0.0	Alphaproteobacteria
J9	1307	JN391168	*Aquimarina* sp.	99	0.0	Bacteroidetes
J10	1256	JN391169	*Bermanella* sp.	95	0.0	Alphaproteobacteria
J11	1252	JN391170	*Pseudomonas* sp.	99	0.0	Gammaproteobacteria
J12	1288	JN391171	*Cytophaga* sp.	99	0.0	Bacteroidetes
J13	1190	JN391172	*Pseudomonas* sp.	97	0.0	Gammaproteobacteria
J14	1217	JN391173	*Pseudomonas* sp.	99	0.0	Gammaproteobacteria
J15	1265	JN391174	*Cytophaga* sp.	96	0.0	Bacteroidetes
J16	244	JN391175	*Paracoccus* sp.	99	1e-122	Alphaproteobacteria
J17	1315	JN391176	*Pseudoalteromonas* sp.	99	0.0	Gammaproteobacteria
J18	796	JN391177	*Pseudoalteromonas* sp.	98	0.0	Gammaproteobacteria
J19	1274	JN391178	*Photobacterium* sp.	96	0.0	Gammaproteobacteria

Rhizobiaceae (J8). The second most represented phylogenetic group was Bacteroidetes representing 21% of the epiphytic totality and belonging to three genera: *Flavobacteria*, *Aquimarina* and *Cytophaga*. However, only one strain was found assigned to the Firmicutes and was identified as closely related to *Bacillus*. These data demonstrate that the *J. rubens*-associated bacterial community was diverse with Simpson's index D of 0.035. Phylogenetic analysis of the 16S rRNA gene sequences of the *J. rubens* bacterial isolates was performed using the Neighbor Joining method as available in MEGA4.0 (Tamura et al. 2004) with 1000 replications. Most of the members of the Alphaproteobacteria group formed one clade with a bootstrap value of 48%. Members of group Bacteroidetes, i.e. J9, J6, J12, J15 and J19, cluster in same clade with strong bootstrap value (74%) as shown in Fig. 1.

J. rubens-identified isolates were subsequently tested for their antimicrobial potential against 19 pathogens. The antimicrobial assay showed that amongst isolates, seven strains corresponding to about 36% were active against pathogens with different antimicrobial spectrum (Table 3). These active isolates coded J2, J9, J11, J13, J16, J17 and J18 were assigned to five genera: *Bacillus*, *Aquimarina*, *Pseudomonas*, *Pseudoalteromonas* and *Paracoccus*. Three of them, J2, J9 and J13, identified respectively as *Bacillus pumilus*, *Aquimarina* sp. and *Pseudomonas* sp. showed the strongest inhibition especially against *S. aureus*, *Micrococcus* and *C. albicans*, with inhibition diameters of 25 to 35 mm. The largest antimicrobial spectrum was obtained for J2 which inhibited about 68% of the pathogens, followed by J13 and J11 strains which inhibited 52% and 42% of the tested pathogens respectively.

The three strains of *S. aureus* were the most sensitive, since they were inhibited by J2, J9, J11, J13 and J17 isolates, while *E. faecalis* and *Streptococcus* sp. were only inhibited by J2. However, *E. coli* (three strains) and *P. fluorescens* AH2 were resistant towards all *J. rubens* isolates. Figure 2a, b shows antibacterial activity of J2, J9, J11, J13 and J17 against *S. aureus* by drop method and antibacterial activity of J2 isolates against *Streptococcus* sp. respectively.

Strains J16, J17 and J18 were weakly active, and each one inhibited only four of the 19 tested pathogens. Despite its weak antimicrobial spectrum, J16 isolate identified as *Paracoccus* sp. displayed strong activity against *P. cepacia*. The growth of *S. typhimurium* was inhibited by the two isolates coded J2 and J18.

In order to verify the close relationship between host and isolated bacteria, toxicity of *J. rubens* organic extracts (polar and non-polar) was tested against 18 human and fish pathogens, the yeast *C. albicans*, four bacteria isolated from *J. rubens* surrounding water and against the 19 surface epibionts (J1–J19). Antimicrobial

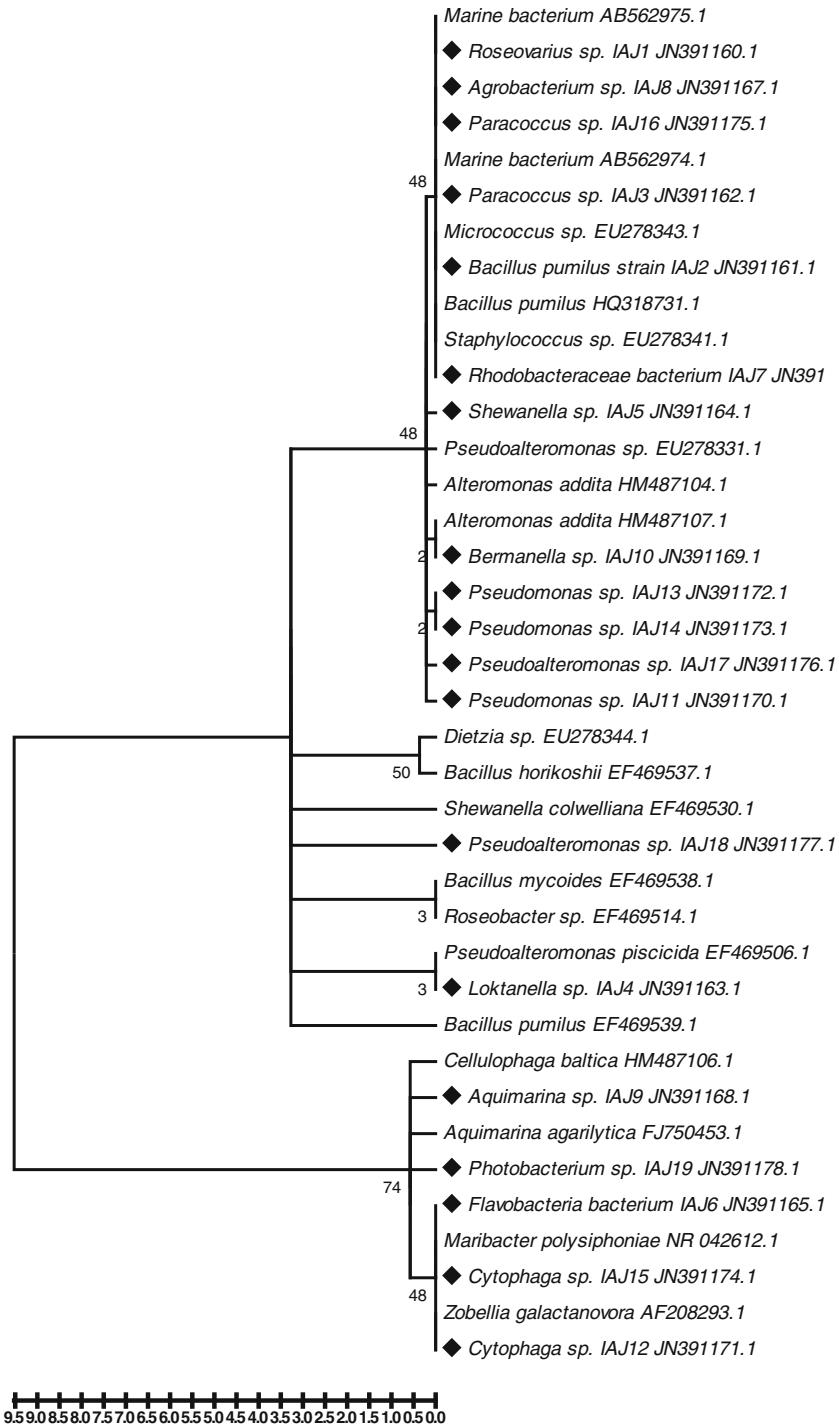

Fig. 1 Neighbor-joining tree based on partial 16S rRNA gene sequences derived from *J. rubens* bacterial isolates and those derived from other red alga isolates (identified via a BLAST search). The *scale bar* indicates 20% of sequence variation

tests revealed activity of *J. rubens* extracts against the pathogens *S. aureus*, *Micrococcus* sp., *Streptococcus* sp. and *P. cepacia*, as well as one of the four surrounding water isolates which was identified by 16S rRNA gene sequence as closely related to *Shewanella* sp. Because the extracts of *Jania* did not inhibit the growth of any of the strains isolated from the surface of this organism, we conceived that the *Jania* isolates were intimately associated with their host.

Discussion

According to the present study, *J. rubens* harbors a diverse community of bacteria associated with its surface of which its members are able to grow on marine agar. 16S rRNA gene sequence analyses were used for bacteria identification.

In bacterial taxonomy it is commonly accepted that two bacteria do belong to the same species unless the 16S rRNA gene sequence similarity is < 97% (Stackebrandt and Goebel

Table 3 Antibacterial spectra of *Jania rubens*-associated bacteria

Test strains	Active isolates						
	J2	J9	J11	J13	J16	J17	J18
Candida albicans	++	+	+	+	+	+	−
Micrococcus sp.	++	+	+	++	−	−	−
Staphylococcus aureus	+++	+++	++	+++	−	+	−
Staphylococcus aureus ATCC 25923	++	++	++	++	−	+	−
Staphylococcus aureus ATCC 6538	++	++	++	++	−	+	−
Enterococcus feacalis ATCC 29212	++	−	−	−	−	−	−
Streptococcus sp.	+++	−	−	−	−	−	−
Vibrio alginoliticus ATCC 17749 T	+	−	+	−	+	−	+
Vibrio. anguillarum	+	−	+	−	−	−	−
Vibrio tapetis CECT 4600	−	−	+	+	−	−	−
Aeromonas salmonicida	++	−	−	+	−	−	+
Aeromonas hydrophila	+	−	−	+	−	−	+
Pseudomonas cepacia	−	−	−	+	+++	−	−
Pseudomonas aeruginosa ATCC 27853	+	−	−	+	+	−	−
Salmonella typhimurium	+	−	−	−	−	−	+

Inhibition zones from 15 to 25 mm were declared as strong (+++), from 7 to 15 as moderate (++) and < 7 as weak activities (+), no activity (−)

1994; Hagström et al. 2000; Cabaj et al. 2006). 16S rRNA gene sequence of strains J10, J15 and J19 displayed a similarity with other published sequences of 95% for J10 and 96% for J15 and J19, which suggests that these are novel species, hitherto unknown species. This has still to be confirmed by further studies; universally conserved protein-coding genes such as gyrB, rpoB, recA and lepA may be used as an alternative to 16S rRNA gene sequencing.

The *J. rubens* bacterial community was considered as diverse according to Simpson's index value. We notice that a number of bacteria associated with other algae and other marine macroorganisms were amongst the closest relatives of phylotypes associated with *J. rubens* (Sakami et al. 1999; Staufenberger et al. 2008).

Flavobacterium spp. were also isolated from *Jania* spp., from Tahiti (Sakami et al. 1999).

In previous investigations (Ismail-Ben Ali et al. unpublished data), we isolated bacteria of the genera *Pseudomonas*, *Pseudoalteromonas*, *Paracoccus* and *Bacillus* from brown alga *Padina pavonica* sampled from the same locality as *J. rubens* (Cap Zebib). Moreover, we reported isolates from *Ulva rigida* surfaces belonging to the genera *Rhodobacteraceae*, *Roseovarius*, *Bacillus*, *Paracoccus*, *Loktanella*, *Shewanella* and *Pseudomonas*. Bacteria of the genus *Loktanella* was also isolated from *Ulva intestinalis* surface (Ismail-Ben Ali et al. 2010). Further studies dealt with the interaction of cultured bacteria with the brown alga *Laminaria* spp. Members of the genera *Flavobacterium* and *Pseudomonas*, with the ability to utilize algal compounds such as mannitol, alginate and laminaran as substrates were isolated (Staufenberger et al. 2008). Bacteria that belong to *Cytophaga-Flavobacterium-Bacteriodes* (CFB) are often found in natural microbial communities in marine environments (Nedashkovskaya et al. 2003). These bacteria play an important role in the normal development of green algae in the marine coastal environment (Matsuo et al. 2003). In addition, members of the CFB group were abundant as

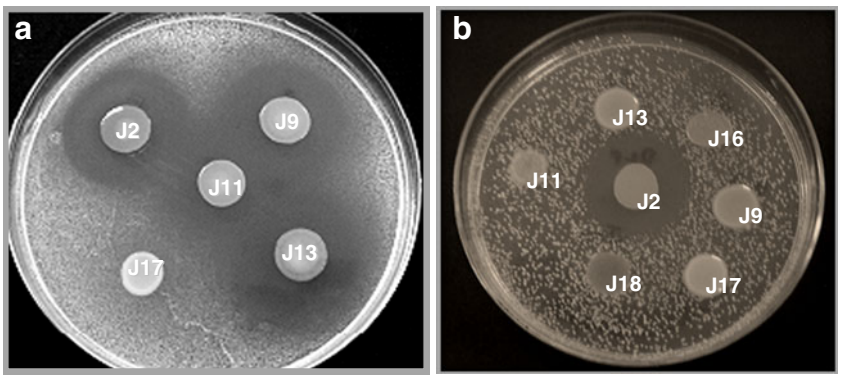

Fig. 2 **a** Antibacterial activity of J2, J9, J11, J13 and J17 against *Staphylococcus aureus* by drop method. **b** Antibacterial activity of J2 isolates against *Streptococcus* sp

epiphytic bacteria on two common freshwater macrophytes, the macroalga *Chara aspera* and the angiosperm *Myriophyllum spicatum* (Hempel et al. 2008).

In our study, identification of *Cytophaga* strain indicates that *J. rubens* harbors bacteria that are important for thallus structural maintenance as reported by Tujula (2006). Matsuo et al. (2003) identified *Cytophaga* species capable of inducing morphogenesis on the green marine alga *Monostroma oxyspermum*, demonstrating the importance of these associated strains in algal morphology.

Strains of the *Cytophaga* and *Pseudomonas* group were previously reported associated with coralline algal surface (Lewis et al. 1985). In addition, algicidal bacteria belonging to the genera *Alteromonas*, *Pseudoalteromonas* and *Cytophaga* were isolated from *Ulva* sp. and *Gelidium* sp. from the coast of Osaka Bay, Japan and have been also reported in coastal red tide areas (Imai et al. 2006).

The marine bacteria *Pseudomonas* sp. and *Pseudoalteromonas* sp. were isolated from the seaweed *Diginea* sp. and the sponge *Halisarca ectofibrosa* (Rungprom et al. 2008; Dahiya and Gautam 2011). Similarly, Bernan et al. (1997) isolated *Pseudomonas* sp. from the surface of red algae.

Members of the *Roseobacter* clade are abundant and widespread in marine habitats (Gonzalez and Moran 1997; Rappé et al. 2000; Zubkov et al. 2001; Selje et al. 2004; Martens et al. 2007). Organisms of this group are also associated with cephalopods (Barbieri et al. 2001) or algae (Shiba 1991; Lafay et al. 1995; Ashen and Goff 2000; Grossart et al. 2005). Shiba (1991) isolated *Roseobacter litoralis* and *R. denitrificans* from surfaces of green seaweeds. Also, as reported by Wang et al. (2009), bacteria of *Roseovarius* genus have been isolated from sediment from South China Sea. Ruiz-Ponte et al. (1999) and Wagner-Dobler et al. (2004) reported antibiotic activity from some species of *Roseobacter*, did not agree with our observations since strain J1 identified as closely related to *Roseovarius* was found inactive against tested pathogens. It may have **another** role as reported by **others who proposed** that members of the *Roseobacter* lineage play a key role in dimethylsulfoniopropionate (DMSP) cleavage and demethylation/demethiolation and correlated their presence and activity on algal surfaces with DMSP-producing algae (Gonzalez and Moran 1997). In this study, we isolated members of genus *Agrobacterium* from *J. rubens* surface; in previous investigation, five species of genus *Agrobacterium* were isolated from northeastern Atlantic Ocean bottom sediments by Rüger and Höfle (1992). Misawa and Shimada (1998) isolated the *crt* gene clusters responsible for the biosynthesis of carotenoids, from the marine bacterium *Agrobacterium aurantiacum.*

Shewanella species represent one of the most numerically abundant microorganisms among readily cultivated marine proteobacteria (Ivanova et al. 2003). Members of this genus have been studied extensively because of their important role in co-metabolic bioremediation of halogenated organic pollutants (Petrovskis et al. 1994), destructive souring of crude petroleum (Semple and Westlake 1987), the dissimilatory reduction of manganese and iron oxides (Myers and Nealson 1988), and their ability to produce high amounts of polyunsaturated fatty acids (Russell and Nichols 1999). Ivanova et al. (2003) reported and characterized new bacteria of the genus *Shewanella* (*Shewanella waksmanii*) isolated from the marine sipuncula. In other investigation, Ivanova et al. (2004) characterized *Shewanella pacifica*, a polyunsaturated fatty acid-producing bacterium isolated from seawater.

Bacteria of genera *Pseudomonas*, *Pseudoalteromonas*, *Bacillus*, *Roseovarius* and *Cytophaga* have been consistently isolated from marine algae, while bacteria of the genera *Paracoccus*, *Shewanella* and *Cytophaga* were occasionally isolated from algae surfaces, whereas compared to previous investigations, bacteria belonging to *Aquimarina* genus are rarely identified from algae and seem to be specific to *J. rubens* epiphytic communities. Hengst et al. (2010) reported epiphytic bacterial communities living on intertidal seaweeds at the northern coast of Chile and identified bacteria belonging to *Aquimarina*, using clonal approach. Nedashkovskaya et al. (2005) described a novel *Aquimarina muelleri* isolated from seawater. Similarly, J10 isolate was identified as closely related to *Bermanella*, was for the first time isolated from *J. rubens* surface. The novel gammaproteobacterium *Bermanella marisrubri*, designed strain RED65^{T}, was isolated from the Red Sea at a depth of 1 m and its genome was sequenced (Pinhassi et al. 2009).

Results of *J. rubens* isolates screened for antimicrobial potentialities highlighted that bacteria of the genera *Bacillus*, *Aquimarina*, *Pseudomonas*, *Pseudoalteromonas* and *Paracoccus* were active against pathogens with a varying and wide antimicrobial spectrum. These findings suggest a beneficial relationship between algae and these epiphytic bacteria which may be involved in preventing fouling. Previous studies have found novel compounds possessing antibiotic activities have been identified from several seaweed-associated bacteria. Our results agree with previous research in which *Pseudomonas*, *Pseudoalteromonas* and *B. pumilus* species isolated from *Laminaria saccharina* were found to produce antibacterial substances (Wiese et al. 2009). Similarly, *Pseudoalteromonas tunicata* is known as a successful competitor on marine surfaces due to its ability to produce a number of inhibitory substances. As such *P. tunicata* has become the model organism for studies of surface colonization and eukaryotic host–bacteria interactions (Thomas et al. 2008).

A natural cyclotetrapeptide cyclo-(isoleucyl-prolyl-leucyl-alanyl) has been isolated from the marine bacteria *Pseudomonas* sp. and *Pseudoalteromonas* sp., associated with the seaweed *Diginea* sp. and the sponge *H. ectofibrosa* (Rungprom et al. 2008; Dahiya and Gautam 2011) and displayed antibacterial activity against *P. aeruginosa* and *K. pneumoniae*, and antifungal activity against pathogenic *C. albicans* (Dahiya and Gautam 2011). Algicidal bacteria belonging to the genera *Alteromonas*, *Pseudoalteromonas* and *Cytophaga* were also isolated from *Ulva* sp. and *Gelidium* sp. Kamei and Isnansetyo (2003) reported lysis of methicillin-resistant *S. aureus* by 2,4-diacetylphloroglucinol produced by marine algal associated species *Pseudomonas* sp.

Similarly, Bernan et al. (1997) mentioned that surugatoxin, tetrodotoxin and anhydrotetrodotoxin have been isolated from a microbial source and some of these microbial sources include a *Pseudomonas* sp. isolated from the surface of red algae. Yasumoto et al. (1986) mentioned that tetrodotoxin (TTX) is produced as a fermentation product of *Pseudomonas* sp. that was isolated from *Jania* sp.

Furthermore, marine *Bacillus* isolates have been found to be able to produce peptide compounds with antimicrobial activity (Jaruchoktaweechai et al. 2000; Barsby et al. 2001; Hentschel et al. 2001). Okarni et al. (1980) discovered a new enzyme that degrades the glucan of *Streptococcus mutans*, which is the cause of dental caries. The enzyme was isolated from a marine *Bacillus* and showed optimum activity at 37°C, which makes it favorable for use in the oral cavity. Burgess et al. (2003) isolated several bacteria with high antifouling activity and found that most of these bacteria belonged to *Bacillus*, such as *B. pumilus*, *B. licheniformis* and *B. subtilus*. Kanagasabhapathy et al. (2006) have isolated *B. pumilus* and other *Bacillus* species from different brown alga. Kanagasabhapathy et al. (2008) investigated antimicrobial activity of epiphytic bacteria from several red algae and found that the highest activity was produced by certain *Bacillus* species especially *B. cereus* and *B. pumilus*. Our results are in agreement with these studies since *B. pumilus* was found active against *S. aureus*, *Vibrio* spp. and *S. typhimurium*.

While it is known that several epiphytic bacteria lose their ability to produce antimicrobial compounds after many subcultures on artificial growth media, in the present report all active isolates, except J9, preserved their abilities to produce inhibitory substances against sensitive pathogens after successive subcultures on marine agar giving the same inhibition of growth of pathogens. However, as mentioned by Penesyan et al. (2010), marine surface-associated microorganisms may require conditions that resemble their native environment in order to produce the maximum amount of bioactives, which is the case for J9, which showed a decrease of activity when grown after several transfers on marine agar. This species may require different growth conditions for optimal production of desired metabolites.

Jania rubens harbors a high diversity of epiphytic bacteria on its surface. In the aim to determine the degree of affinity of *J. rubens* epibionts to their host, we tested inhibition of *Jania* organic crude extracts against all isolated bacterial species as well as against human and fish pathogens and bacteria isolated from the surrounding water. Results revealed activity of *J. rubens* extracts against the pathogens *S. aureus*, *Micrococcus* sp., *Streptococcus* sp. and *P. cepacia* as well as one of the four surrounding water isolates identified as *Shewanella* sp., while both dichloromethane and dichloromethane/methanol crude extracts were inactive towards all *Jania* isolates. Other investigations agree with our finding, and antimicrobial activity of *J. rubens* has been previously reported; the aqueous extract of *J. rubens* showed high antibacterial activity against *B. subtilis* and a low activity against *S. aureus* (Soliman et al. 1994). Similarly, Karabay-Yavasoglu et al. (2007) found that *J. rubens* extracts, especially methanol and chloroform extracts, possess antimicrobial activity. Moreover, volatile constituents, fatty alcohols and hydrocarbon fractions isolated from *J. rubens* displayed varying antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli* (Awad 2002). In view of these results, *J. rubens* epibionts should be intimately associated with their host.

We conclude that the bacteria isolated from the surface of *J. rubens* are closely associated with their host and may represent a new source of antimicrobial secondary metabolites highly active against several Gram-negative and Gram-positive pathogens as well as the yeast *C. albicans*. These epibionts might be beneficial to the algae by limiting or preventing the development of competing or fouling bacteria. Moreover, we reported here an isolate of *Aquimarina* highly active against the pathogens *Staphylococcus*, *Micrococcus* and *C. albicans* pathogens and which is rarely isolated from red algae and newly isolated from *J. rubens* surface.

Acknowledgements The authors thank Mrs. Veronique Confurius-Guns, Department of Marine Microbiology, Netherlands Institute of Ecology, NIOO-KNAW, Yerseke, The Netherlands, for her assistance and help with PCR and DNA sequencing. This is NIOO-KNAW publication 5145.

References

Armstrong E, Rogerson A, Leftley JW (2000) The abundance of heterotrophic protists associated with intertidal seaweeds. Est Coast Shelf Sci 50:415–424

Ashen JB, Goff LJ (2000) Molecular and ecological evidence for species specificity and coevolution in a group of marine algal–bacterial symbioses. Appl Environ Microbiol 66:3024–3030

Awad NE (2002) Biologically active constituents from the red alga *Jania rubens* (L) Lamx. Bull Fac Pharm 40:169–174

Barbieri E, Paster BJ, Hughes D, Zurek L, Moser DP, Teske A, Sogin ML (2001) Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda: *Loliginidae*). Environ Microbiol 3:151–167

Barott KL, Rodriguez-Brito B, Janouškovec J, Marhaver KL, Smith JE, Keeling P, Rohwer FL (2011) Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral *Montastraea annularis*. Environ Microbiol 13:1192–1204

Barsby T, Kelly MT, Gagne SM, Andersen RJ (2001) Bugorol A produced in culture by a marine *Bacillus* sp. reveals a novel template for cationic peptide antibiotics. Org Lett 3:437–440

Bernan VS, Greenstein M, Maiese WM (1997) Marine microorganisms as a source of new natural products. Adv Appl Microbiol 43:57–90

Boyd KG, Adams DR, Burgess JG (1999) Antibacterial and repellent activities of marine bacteria associated with algal surfaces. Biofouling 14:227–236

Burgess JG, Boyd KG, Armstrong E, Jiang Z, Yan L, Berggren M, May U, Pisacane T, Granmo A, Adams DR (2003) The development of a marine natural product-based antifouling paint. Biofouling 19:197–205

Cabaj A, Palińska K, Kosakowska A, Kurlenda J (2006) Heterotrophic bacteria from brackish water of the southern Baltic Sea: biochemical and molecular identification and characterization. Oceanologia 48:525–543

Cheng XC, Jensen PR, Fenical W (1999) Luisols A and B, new aromatic tetraols produced by an estuarine marine bacterium of the genus *Streptomyces* (Actinomycetales). J Nat Prod 62:608–610

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438:90–93

Dahiya R, Gautam H (2011) Toward the synthesis and biological screening of a cyclotetrapeptide from marine bacteria. Mar Drugs 9:71–81

Gil-Turnes MS, Hay ME, Fenical W (1989) Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. Science 246:116–118

Gonzalez JM, Moran MA (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class Proteobacteria in coastal seawater. Appl Environ Microbiol 63:4237–4242

Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. Environ Microbiol 7:860–873

Hagstrom A, Pinhassi J, Zweifel UL (2000) Biogeographical diversity among marine bacterioplankton. Aquat Microb Ecol 21:231–244

Hempel M, Blume M, Blindow I, Gross EM (2008) Epiphytic bacterial community composition on two common submerged macrophytes in brackish water and freshwater. BMC Microbiol 8:58

Hengst MB, Andrade S, González B, Correa JA (2010) Changes in epiphytic bacterial communities of intertidal seaweeds modulated by host, temporality, and copper enrichment. Microb Ecol 60:282–290

Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. FEMS Microbiol Ecol 35:305–312

Holmstrom C, Egan S, Franks A, McCloy S, Kjelleberg S (2002) Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. FEMS Microbiol Ecol 41:47–58

Imai I, Fujimaru D, Nishigaki T, Kurosaki M, Sugita H (2006) Algicidal bacteria isolated from the surface of seaweeds from the coast of Osaka Bay in the Seto Inland Sea, Japan. Afr J Mar Sci 28:319–323

Imhoff JF, Labes A, Wiese J (2011) Bio-mining the microbial treasures of the ocean: new natural products. Biotechnol Adv 29:468–482

Ismail-Ben Ali A, Ktari L, Bolhuis H, Boudabbous A, Stal LJ, El Bour M (2010) *Ulva intestinalis* associated bacteria: molecular identification and antimicrobial potential. Rapp Comm Int Mer Médit 39:316

Ivanova EP, Nedashkovskaya OI, Zhukova NV, Nicolau DV, Christen R, Mikhailov VV (2003) *Shewanella waksmanii* sp. nov., isolated from a sipuncula (*Phascolosoma japonicum*). Int J Syst Evol Microbiol 53:1471–1477

Ivanova EP, Gorshkova NM, Bowman JP, Lysenko AM, Zhukova NV, Sergeev AF, Mikhailov VV, Nicolay DV (2004) *Shewanella pacifica* sp. nov., a polyunsaturated fatty acid-producing bacterium isolated from sea water. Int J Syst Evol Microbiol 54:1083–1087

James SG, Holmström C, Kjelleberg S (1996) Purification and characterization of a novel antibacterial protein from the marine bacterium D2. Appl Environ Microbiol 62:2783–2788

Jaruchoktaweechai C, Suwanborirux K, Tanasupawatt S, Kittakoop P, Menasveta P (2000) New macrolactins from a marine *Bacillus* sp. Sc026. J Nat Prod 63:984–986

Jiang ZD, Jensen PR, Fenical W (1999) Lobophorins A and B, new antiinflammatory macrolides produced by a tropical marine bacterium. Bioorg Med Chem Lett 9:2003–2006

Johnson CR, Muir DG, Reysenbach AL (1991) Characteristic bacteria associated with surfaces of coralline algae: a hypothesis for bacterial induction of marine invertebrate larvae. Mar Ecol Prog Ser 74:281–294

Kamei Y, Isnansetyo A (2003) Lysis of methicillin-resistant *Staphylococcus aureus* by 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. AMSN isolated from a marine alga. Int J Antimicrob Agents 21:71–74

Kanagasabhapathy M, Sasaki H, Haldar S, Yamasaki S, Nagata S (2006) Antimicrobial activities of marine epibiotic bacteria isolated from brown algae of Japan. Ann Microbiol 56:167–173

Kanagasabhapathy M, Sasaki H, Nagata S (2008) Phylogenetic identification of epibiotic bacteria possessing antimicrobial activities isolated from red algal species of Japan. World J Microbiol Biotechnol 24:2315–2321

Karabay-Yavasoglu NU, Sukatar A, Ozdemir G, Horzum Z (2007) Antimicrobial activity of volatile components and various extracts of the red alga *Jania rubens*. Phytother Res 21:153–156

Konig GM, Wright AD, Stiche O, Angerhofer CK, Pezzuto JM (1994) Biological activities of selected marine natural products. Planta Med 60:532–537

Ktari L, Blond A, Guyot M (2000) 16β-Hydroxy-5α-cholestane-3,6-dione, a novel cytotoxic oxysterol from the red alga *Jania rubens*. Bioorg Med Chem Lett 10:2563–2565

Lafay B, Ruimy R, Traubenberg CR, Breittmayer V, Gauthier MJ, Christen R (1995) *Roseobacter algicola* sp. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. Int J Syst Bacteriol 45:290–296

Lemos ML, Toranzo AE, Barja JL (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microb Ecol 11:149–163

Lewis TE, Garland CD, Thomas A (1985) The bacterial biota on crustose (nonarticulated) coralline algae from Tasmanian waters. Microb Ecol 11:221–230

Martens T, Gram L, Grossart H-P, Kessler D, Muller R, Simon M, Wenzel SC, Brinkhoff T (2007) Bacteria of the *Roseobacter* clade show potential for secondary metabolite production. Microb Ecol 54:31–42

Matsuo Y, Suzuki M, Kasai H, Shizuri Y, Harayama S (2003) Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. Environ Microbiol 5:25–35

Mearns-Spragg A, Boyd KG, Bregu M and Burgess JG (1998) Cross species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates after exposure to terrestrial bacteria. Lett Appl Microbiol 27:142–146

Misawa N, Shimada H (1998) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J Biotechnol 59:169–181

Myers CR, Nealson KH (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science 240:1319–1321

Nedashkovskaya OI, Kim SB, Han SK, Lysenko AM, Rohde M, Zhukova NV, Falsen E, Frolova GM, Mikhailov VV, Bae KS (2003) *Mesonia algae* gen. nov., sp. nov., a novel marine bacterium of the family Flavobacteriaceae isolated from the green alga *Acrosiphonia sonderi* (Kütz) Kornm. Int J Syst Evol Microbiol 53:1967–1971

Nedashkovskaya OI, Kim SB, Lysenko AM, Frolova GM, Mikhailov VV, Lee KH, Bae KS (2005) Description of *Aquimarina muelleri* gen. nov., sp. nov., and proposal of the reclassification of [*Cytophaga*] *latercula* Lewin 1969 as *Stanierella latercula* gen. nov., comb. nov. Int J Syst Evol Microbiol 55:225–229

Okarni Y, Kurasawa S, Hirose Y (1980) A new glucanase produced by a marine *Bacillus*. Agric Biol Chem 44:1191–1192

Penesyan A, Kjellberg S, Egan S (2010) Development of novel drugs from marine surface associated microorganisms. Mar Drugs 8:438–459

Petrovskis EA, Vogel TM, Adriaens P (1994) Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1. FEMS Microbiol Lett 121:357–364

Pinhassi J, Pujalte MJ, Pascual J, Gonzalez JM, Lekunberri I, Pedros-Alio C, Arahal DR (2009) *Bermanella marisrubri* gen. nov., sp. nov., a genome-sequenced gammaproteobacterium from the Red Sea. Int J Syst Evol Microbiol 59:373–377

Rao D, Webb JS, Kjelleberg S (2005) Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*. Appl Environ Microbiol 71:1729–1736

Rao D, Webb JS, Kjelleberg S (2006) Microbial colonization and competition on the marine alga *Ulva australis*. Appl Environ Microbiol 72:5547–5555

Rao D, Webb JS, Holmstrom C, Case R, Low A, Steinberg P, Kjelleberg S (2007) Low densities of epiphytic bacteria from the marine alga *Ulva australis* inhibit settlement of fouling organisms. Appl Environ Microbiol 73:7844–7852

Rappé MS, Vergin K, Giovannoni SJ (2000) Phylogenetic comparisons of a coastal bacterioplankton community with its counterparts in open ocean and freshwater systems. FEMS Microbiol Ecol 33:219–232

Ruiz-Ponte C, Samain JF, Sanchez JL, Nicolas JL (1999) The benefit of a *Roseobacter* species on the survival of scallop larvae. Mar Biotechnol 1:52–59

Rungprom W, Siwu ERO, Lambert LK, Dechsakulwatana C, Barden MC, Kokpol U, Blanchfield JT, Kita M, Garson MJ (2008) Cyclic tetrapeptides from marine bacteria associated with the seaweed *Diginea sp*. and the sponge *Halisarca ectofibrosa*. Tetrahedron 64:3147–3152

Russell NJ, Nichols DS (1999) Polyunsaturated fatty acids in marine bacteria—a dogma rewritten. Microbiology 145:767–779

Rüger H-J, Höfle MG (1992) Marine star-shaped-aggregate-forming bacteria: Agrobacterium atlanticum sp. nov.; Agrobacterium meteori sp. nov.; Agrobacterium ferrugineum sp. nov., nom. rev.; Agrobacterium gelatinovorum sp. nov., nom. rev.; and Agrobacterium stellulatum sp. nov., nom. rev. Int J Syst Evol Microbiol 42:133–143

Sakami T, Nakahara H, Chinain M, Ishida Y (1999) Effects of epiphytic bacteria on the growth of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). J Exp Mar Biol Ecol 233:231–246

Selje N, Simon M, Brinkhoff T (2004) A newly discovered *Roseobacter* cluster in temperate and polar oceans. Nature 427:445–448

Semple KM, Westlake DWS (1987) Characterization of iron reducing *Alteromonas putrefaciens* strains from oil field fluids. Can J Microbiol 35:925–931

Shiba T (1991) *Roseobacter litoralis* gen. nov., sp. nov., and *Roseobacter denitrificans* sp. *nov.*, aerobic pink-pigmented bacteria which contain bacteriochlorophyll a. Syst Appl Microbiol 14:140–145

Soliman FM, El Tohamy SF, Fathy MM, Ramadan A, Afyfy NA, Sanad OA (1994) Phytochemical and biological investigation of *Jania rubens* (L.) Lamx. amino acids, proteins, nitrogenous bases and biological screening. J Drug Res 21:155–164

Stackebrandt E, Goebel BM (1994) A place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Staufenberger T, Thiel V, Wiese J, Imhoff JF (2008) Phylogenetic analysis of bacteria associated with Laminaria saccharina. FEMS Microbiol Ecol 64:65–77

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Nati Acad Sci USA 101:11030–11035

Thomas T, Evans FF, Schleheck D, Mai-Prochnow A, Burke C, Penesyan A, Dalisay DS, Stelzer-Braid S, Saunders N, Johnson J, Ferriera S, Kjelleberg S, Egan S (2008) Analysis of the *Pseudoalteromonas tunicata* genome reveals properties of a surface-associated life style in the marine environment. PLoS One 3(9):e3252

Tujula NA (2006) Analysis of epiphytic bacterial communities associated with the green alga *Ulva australis*. PhD Thesis, University of New South Wales.

Wagner-Dobler I, Rheims H, Felske A, El-Ghezal A, Flade-Schorder D, Laatsch H, Lang S, Pukall R, Tindall BJ (2004) *Oceanibulbus indolifex* gen. nov., sp. nov., a North Sea *Alphaproteobacterium* that produces bioactive metabolites. Int J Syst Evol Microbiol 54:1177–1184

Wang B, Sun F, Lai Q, Du Y, Liu X, Li G, Luo J, Shao Z (2009) *Roseovarius nanhaiticus* sp. nov., a member of the *Roseobacter* clade isolated from marine sediment. Int J Syst Evol Microbiol 60:1289–1295

Wiese J, Thiel V, Nagel K, Staufenberger T, Imhoff JF (2009) Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. Mar Biotechnol 11:287–300

Yasumoto T, Yasumura D, Yotsu M, Michishita T, Endo A, Kotaki Y (1986) Bacterial production of tetrodotoxin and anhydrotetrodotoxin. Agric Biol Chem 50:793–795

Zubkov MV, Fuchs BM, Burkill PH, Amann R (2001) Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. Appl Environ Microbiol 67:5210–5218