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Antibacterial and biofilm inhibitory activities of bacteria associated with polychaetes

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ABSTRACT

Objective: To study the antibacterial and antibiofilm activities expressed by epibiotic bacteria associated with the polychaetes *Platynereis dumerilii* and *Syllis* sp.

Methods: A total of 32 cultivable bacterial strains were isolated from the two polychaete species. The crude extracts were tested for antibacterial activity and biofilm inhibitory activity against pathogenic and biofilm-forming bacterial strains. Extracts of the strains which showed strong activity were analyzed by thin-layer chromatography (TLC) and the bacterial strains were identified based on 16S rRNA gene sequencing.

Results: Extracts of 13 bacterial strains showed inhibitory activity against pathogenic and biofilm-forming bacteria. The crude extracts also affected the synthesis of extracellular polymeric substances and cell surface hydrophobicity of the *Alteromonas* sp. isolated from marine biofilm. The adhesion of *Alteromonas* sp. on glass surface showed significant variation between surface-associated bacterial crude extract treatment and control groups. Among the 13 bacteria, two strains PA8 and PA19 were further analyzed for bioactive fractions. Thin-layer chromatography indicated the presence of a single active fraction in the crude extract of both the bacterial strains. The epibiotic bacterial strains P8 and P19 were identified as *Exiguobacterium* sp. and *Actinobacterium* sp. respectively based on 16S rRNA gene sequencing.

Conclusions: The present study indicates that bacteria associated with marine invertebrates inhabiting the coastal waters could be used as a potential source for the isolation of bioactive metabolites.

1. Introduction

In the marine environment, macroorganisms are generally colonized by bacterial communities (also called as epibiotic bacteria) from the surrounding waters[1,2]. The colonization of microbes on marine macroorganisms may sometimes reach up to 40-60% of weight of the organism (*e.g.* sponge)[3], or the diversity may be high (many strains in an animal)[4]. The association between invertebrates and microbes occurs for many purposes. For example, these microbes associated with marine organisms may produce secondary metabolites for increasing the chance of their survival in the competitive conditions (defense against antagonists) prevailing on the surface of the marine organisms[5]. In some cases, the compounds produced by the epibiotic bacterial community provide defense to the host from the predators[6]. Epibiotic bacteria are cultivable and have the potential to offer a sustainable supply of desired compounds for pharmacological investigations.

Study of marine microorganisms has gained importance in recent years because they produce novel metabolites, which exhibit antimicrobial, antiviral, antitumor as well as anticoagulant properties[7-9]. Identification of microorganisms that produce novel bioactive compounds is of great interest for the development of new molecules to fight against many pathogens and produce industrially important compounds. Bacteria associated with the surface of marine invertebrates are reported to contain a higher proportion of antibacterial and antifouling activities than those occur as planktonic forms[10]. Previous studies mainly focused on the bacteria associated with sponges, corals and seaweeds and tested for their antimicrobial activity[2,11-16] and many novel bioactive metabolites have been reported[17-19]. However, only a few works are available on the bioactivity of epibiotic bacterial communities associated with other invertebrate groups including polychaetes which are considered as a dominant group in benthic communities.

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Though bioactive metabolites from marine organisms are mainly searched for biomedical applications, considerable attention has been paid for screening antifouling compounds[20,21]. Bacterial adhesion on surfaces and subsequent development of biofilms are major problems in aquatic environments. In the marine environment, biofilm development on surfaces of technical objects leads to the recruitment of biofouling communities that warrant frequent cleaning or application of antifouling biocides. Biotechnological approach to develop non-toxic or less toxic bioactive antifoulants gained significance in recent past due to the environmental concern associated with the application of chemobiocides to control biofouling. Natural products are considered as promising alternatives to conventional antifouling systems. The secondary metabolites produced by marine microbes, particularly associated with living and non-living surfaces, express strong antimicrobial and also antifouling activities against micro- and macrofouling organisms[22,23]. Since only a minor proportion of microorganisms associated with marine organism has been screened for the production of antifouling compounds, it is necessary to focus more research to utilize their bioactive potential. In the present study, cultivable bacteria associated with two polychaete species were screened for the antibacterial and antibiofilm activities. The main objective is to investigate the antimicrobial and biofilm inhibitory activities of bacteria associated with polychaete species and to identify bioactive bacterial strains through 16S rRNA gene sequencing. Results obtained will improve our knowledge on the bioactivity of bacterial communities associated with marine invertebrates and also provide leads for the development of drugs and antibiofilm compounds.

2. Materials and methods

The polychaetes, *Syllis* sp. and *Platynereis dumerilii* were collected from the intertidal region of the Kanyakumari coastal waters (Southeast coast of India). The collected polychaete samples were kept in an icebox and brought to the laboratory for the isolation of associated bacteria. In the laboratory, the polychaete samples were gently washed with sterile seawater to remove the loosely attached organisms and other epifauna. The viable heterotrophic bacteria associated with the polychaete species were obtained by cotton swabbing. The cotton swabs were then placed in 2 mL sterile seawater and vortexed. The resulting suspension was serially diluted and an aliquot of the appropriate dilution was inoculated into Zobell marine agar (Himedia, India) plates. All the plates were incubated for a period of 24-48 h for the development of bacterial colonies. The developed colonies were isolated based on colony morphology and purified by repeatedly streaking on Zobell marine agar plates. The purified colonies were maintained in agar slants at 4 °C. The isolated strains were characterized based on morphology, motility, Gram-staining, gelatin and starch hydrolysis, catalase and oxidase activity, nitrate reduction, citrate utilization, indole production, MR-VP and various sugar tests (according to Bergey's Manual of Determinative Bacteriology).

2.1. Preparation of crude extracts of polychaete-associated bacteria

The bacterial strains isolated from the surface of polychaete species (hereafter also mentioned as surface-associated bacteria) were inoculated into Zobell marine broth in 250 mL conical flasks. The flasks were incubated at room temperature with a little agitation (100 r/min) for 7 days. The 7-day-old culture broth was used to prepare the crude extracts. The culture broth was centrifuged at 9 700 r/min for 15 min at 4 °C. The pellet and supernatant were collected separately. The cell pellet was washed with phosphate buffered saline (10 mmol/L) and used for further extraction. Methanol was used as the solvent for the extraction of metabolites from cell pellets. The cell pellets were extracted with 2 mL of methanol and the supernatant was concentrated to get dry extract powder. This dried extract was mixed with a known volume of methanol (1 mg in 1 mL methanol) and used for the antimicrobial activity screening.

2.2. Screening of antimicrobial activity of surface-associated bacterial extracts

The antimicrobial activity of the crude extracts was studied by disc diffusion assay. *Bacillus subtilis*, *Aeromonas* sp., *Klebsiella* sp. and *Vibrio* sp. were the pathogens used as test organisms for the antimicrobial activity. Similarly, three bacterial strains *Pseudomonas* sp., *Alteromonas* sp. and *Gallionella* sp. isolated from the marine biofilm were also used as target strains. For the disc diffusion assay, 50 μL of the extract (1 mg dry extract in 1 mL solvent) was loaded on the paper disc (Whatman-filter paper, 6 mm, Himedia). The extract was allowed to saturate throughout the disc and placed on nutrient agar plates seeded with target bacteria. Zobell marine agar plates were used for the biofilm bacterial strains. The plates were incubated for 24-48 h at room temperature. The zones of inhibition, if any, around the disc were measured. The discs loaded with methanol were used as control.

2.3. Preparation of target bacterial cell suspension

Alteromonas sp. (biofilm-forming bacterial strain) was used as the test organism for the adhesion assay, hydrophobicity and extracellular polymeric substances (EPS) production studies. This bacterial strain was isolated from the marine biofilm developed on acrylic coupons submerged in the coastal waters and maintained in our laboratory (Center for Marine Science and Technology, Manonmaniam Sundaranar University, India) for antifouling screening studies of natural product[2,24]. The bacterial strain was inoculated in 250 mL conical flasks having Zobell marine broth (HIMEDIA, India). The culture broth was incubated for a period of 48 h at room temperature. After the period of incubation, the bacterial culture broth was centrifuged at 5 000 r/min for 15 min. The cell pellets obtained after centrifugation were washed with phosphate buffer saline (10 mmol/L) and re-suspended in the same buffer to obtain optical density (OD) at 540 nm of 0.2. The cell suspension was used for further studies.

2.4. Influence of polychaete-associated bacterial extracts on EPS production of the target bacteria

This experiment was carried out to assess the influence of surface-associated bacterial extracts on the EPS production by the target bacteria. The *Alteromonas* sp. cell suspension as prepared above (3 mL) was taken in a test tube. One milliliter of crude surface-associated bacterial extracts was added to the test tubes and incubated for 24 h at room temperature. The test tubes containing 3 mL *Alteromonas* sp. cell suspension and 1 mL methanol were considered as control. After the period of incubation, the bacterial cell suspension was centrifuged at 9 700 r/min for 15 min at 4 °C. The supernatant was collected in a vial

and equal volume of ethanol was added. These vials were kept for 24 h at room temperature for the precipitation of EPS. The precipitated EPS was collected separately and washed with distilled water through a membrane filter (0.45 μm). The filtrate was dialyzed against the distilled water to remove the impurities. The concentration of proteins and carbohydrates of the EPS was quantified using standard methods. Carbohydrate was estimated by modified phenol sulphuric acid method by using glucose as standard[25]. The total protein concentration was estimated by the modified Lowry *et al*.[26] method by using bovine serum albumin as the standard.

2.5. Measurement of hydrophobicity by bacterial adherence to hydrocarbons (BATH) assay

The hydrophobicity of the bacterial cell suspension was evaluated by using BATH assay following the procedure described by Rosenberg[27]. In brief, 3 mL of bacterial cell suspension (*Alteromonas* sp.) was taken in a test tube and 500 μL of surface-associated bacterial crude extracts were added. The bacterial cell suspension with the addition of 500 μL methanol (without surface-associated bacterial crude extracts) was considered as control. The tubes were incubated for 24 h. After the incubation period, 100 μL of hexane was added to the bacterial cell suspension. The suspension was vortexed for 1 min to ensure the mixing of the hexane and bacterial cell suspension, and left to stand for 15 min to allow the separation of two phases. The OD of the aqueous phase was taken at 400 nm by using spectrophotometer. The percentage of the cells bound to *n*-hexane was calculated by the formula:

Bacterial cell adherence to hexane = (1 - A/Ao) × 100

Where, Ao was the OD of the aqueous phase before adding *n*-hexane and A was the OD after the addition of *n*-hexane.

2.6. Adhesion assay

The experiment consists of two steps, (1) treatment of biofilm bacterial cells with crude extracts of bacteria isolated from the polychaetes and (2) adhesion assay by using the crude extracts treated and control biofilm bacteria. In brief, 3 mL *Alteromonas* sp. cell suspension was taken in a test tube along with 1 mL crude extracts (1 mg dried extracts in 1 mL methanol). The test tubes without the addition of crude bacterial extracts but containing 1 mL methanol were considered as control. The test tubes were incubated for 2 h at room temperature inside a sterile chamber. After the period of incubation, the *Alteromonas* sp. treated with surface-associated bacterial extracts and control was used for adhesion assay. Bacterial adhesion assay was carried out in 500 mL glass beakers by using glass slides (7.5 × 2.5 cm) as substratum (coupons). Before the experiment, all the glassware were washed with detergent and autoclaved. The glass beakers were filled with 300 mL sterile seawater and added with the *Alteromonas* sp. treated with surface-associated bacterial extracts. Control beakers consist of *Alteromonas* sp. treated with 1 mL methanol. Five glass slides were placed inside the beakers in slanting position for the formation of biofilm. All the beakers were covered with parafilm and kept inside a sterile chamber (UV treated) at room temperature. The coupons were retrieved from the beakers after 1, 3, 6, 9 and 12 h of incubation. The retrieved coupons were air dried, heat fixed and stained with crystal violet. The number of bacterial cells adhered on both control and experimental coupons was counted under the microscope. The experiments were repeated (n = 5) and the mean ± standard deviation values were taken.

2.7. Analysis of surface-associated bacterial extracts by thin-layer chromatography (TLC)

TLC was carried out with the crude extracts on glass plates coated by silica gel (HIMEDIA, India). *N*-butanol, acetone and water (4:5:1) were used as solvent system for TLC. The crude extracts (30 μL) were spotted on the silica gel plates, and the solvent front was allowed to run for approximately 16 cm. The plates were removed from the TLC jar after the solvent reached the end point. Plates were air dried and kept in a chamber with iodine crystals for the detection of active compounds. The R_f value of the distinct spots on the TLC plates was calculated by the formula.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

2.8. Recovery of active fraction from the thin-layer chromatogram

The distinct spots observed on the TLC plates were scraped (around 2.5 cm) into micro-centrifuge tubes and extracted with methanol. The silica gel residue was removed by centrifugation at 10 000 r/min for 15 min. The supernatant was transferred to a second set of centrifuge tubes. The individual metabolites were again spotted on TLC plates for the confirmation of metabolites. Each fraction was concentrated by evaporating the methanol and tested for antibacterial activity. The antibacterial activity test was conducted by disc assay as described above. In brief, 50 μL of the TLC resolved fraction was loaded on the disc and placed on the agar plates seeded with target bacterial strains.

2.9. Identification of bacterial strains

The bacterial strains which showed strong antibacterial and antibiofilm activity were identified by sequencing the 16S rRNA gene. The methods for DNA isolation, PCR amplification and sequence analysis used in our laboratory were reported previously[2] and the sequences were submitted to NCBI GenBank.

2.10. Statistical analysis

The data obtained from the adhesion assay were subjected to Two-way ANOVA (analysis of variance) by using treatments and time as factors. The statistical analysis was performed by MS-Excel program.

3. Results

3.1. Antimicrobial activity of crude extracts of bacteria associated with polychaetes

The crude extracts isolated from 32 bacterial strains were tested for their antimicrobial activity against all the eight target bacterial strains. Among these, 13 strains were found to be active against the test bacteria (Table 1). More than 75% of the strains showed inhibitory activity against all the eight strains with a maximum inhibition zone of 19 mm diameter against *Alteromonas* sp. and 18 mm against *Gallionella* sp. A minimum zone of 7 mm was observed in strain PA9 against *Vibrio harveyi* (*V. harveyi*). The extracts isolated from strain PA9 showed activity against all eight target strains with a maximum inhibition zone of 19 mm diameter against *Alteromonas* sp., 16 mm against *Gallionella* sp. and 15 mm against *Klebsiella* sp. and a minimum inhibitory zone

Table 1

Antibacterial activity of crude extracts isolated from the bacterial strains associated with polychaetes against target bacteria (mm).

Strains	Diameter of zone of inhibition							
	Gallionella sp.	*Pseudomonas* sp.	*Alteromonas* sp.	*V. harveyi*	*Vibrio parahaemolyticus*	*Aeromonas* sp.	*Bacillus subtilis*	*Klebsiella* sp.
PA1	18	10	16	-	11	-	18	9
PA5	9	-	-	11	9	16	11	11
PA8	18	12	17	9	7	9	12	9
PA9	16	11	19	7	14	11	11	15
PA14	10	-	12	-	10	12	-	10
PA17	12	14	10	-	12	17	10	11
PA19	18	9	17	16	17	17	15	12
PA20	-	18	11	-	17	10	14	16
PA22	16	17	-	-	16	10	10	13
PA24	-		16	11	-	16	-	11
PA27	17	-	19	-	17	17	16	10
PA29	17	13	-	16	-	12	17	16
PA32	16	15	-	-	16	17.9	-	13

of 7 mm was observed against *V. harveyi*. The extracts of strain PA8 showed activity against all the eight target strains with a maximum inhibition zone of 18 mm against *Gallionella* sp. and 17 mm against *Alteromonas* sp. and a minimum inhibition zone of 7 mm was observed against *Vibrio parahaemolyticus*. Similarly, the extracts of strain PA19 showed activity against all the eight target strains with a maximum inhibition zone of 18 mm against *Gallionella* sp. and 16 mm against *V. harveyi* and a minimum inhibitory zone of 9 mm diameter was observed against *Pseudomonas* sp.

3.2. Influence of polychaete-associated bacteria on the EPS production in Alteromonas sp.

The carbohydrate concentration of the EPS synthesized by *Alteromonas* sp. culture treated with strain PA9 extracts was 0.118 mg/mL. This value was considerably low when compared to the control (0.217 mg/mL). The carbohydrate concentration of the EPS synthesized by the *Alteromonas* sp. cultures treated with PA8 extracts was also significantly low (0.109 mg/mL) compared to the control. The EPS collected from the *Alteromonas* sp. culture treated with PA22 extracts showed a carbohydrate concentration of 0.081 mg/mL. The carbohydrate concentration was also reduced considerably when treated with PA9 extracts. The carbohydrate concentration of the EPS in all the other polycheate-associated strains did not reduce considerably (Table 2).

Table 2

Influence of crude extracts of polychaete-associated bacteria on the carbohydrate and protein concentration of EPS synthesized by *Alteromonas* sp. (mg/mL).

Bacterial strains	Carbohydrate	Protein
PA1	0.178	0.185
PA5	0.163	0.172
PA8	0.109	0.113
PA9	0.118	0.139
PA14	0.193	0.145
PA17	0.207	0.211
PA19	0.177	0.198
PA20	0.181	0.215
PA22	0.081	0.073
PA24	0.169	0.185
PA27	0.165	0.188
PA29	0.155	0.163
PA32	0.148	0.179
Control	0.217	0.195

The protein concentration of the EPS also varied considerably when treated with the crude extracts of the bacteria isolated from the polychaetes. The protein concentration of the EPS synthesized by the *Alteromonas* culture was very low (0.073 mg/mL), when treated with PA22 extracts. The *Alteromonas* sp. culture treated with PA14 extracts showed a protein concentration of 0.145 mg/mL in the EPS. The protein concentration of EPS synthesized by the *Alteromonas* sp. culture treated with PA8 extracts was 0.113 mg/mL and when treated with PA9 extracts, the protein concentration was 0.139 mg/mL. The protein concentration of EPS in all the other surface-associated bacterial strains did not reduce considerably (Table 2).

3.3. Effect of polychaete-associated bacterial extracts on the hydrophobicity of target bacteria

The hydrophobicity (%) of *Alteromonas* sp. cells was reduced when treated with the extracts of PA1, PA5, PA17, PA20, PA27, PA29 and PA32. However, hydrophobicity showed an increased value when treated with extracts of strains PA22, PA14 and PA24. The hydrophobicity of the *Alteromonas* sp. was found to be 28.3% when treated with PA9 extracts. That was very low compared to the control (58%). The extracts of strain PA19 also reduced the hydrophobicity to a value of 37.5% (Table 3).

Table 3

Influence of crude extracts of bacterial strains isolated from the polychaetes on the hydrophobicity of *Alteromonas* sp. (%).

Bacterial strains	Experiment
PA1	43.5
PA5	43.2
PA8	39.0
PA9	28.3
PA14	59.0
PA17	48.5
PA19	37.5
PA20	49.3
PA22	59.7
PA24	60.1
PA27	52.0
PA29	53.0
PA32	47.0
Control	58.0

3.4. Influence of polychaete–associated bacterial extracts on the adhesion bacteria

The number of *Alteromonas* sp. adhered on the coupons submerged in the medium which contains cells treated with surface-associated crude extracts of the strain PA8 was 1245 cm^{-2} after 1 h. The number of cells attached on the slides submerged in the control medium (without extract treatment) was 2844 cm^{-2} after 1 h (Figure 1). During the same period (after 1 h), the number of *Alteromonas* sp. cells found on the coupons submerged in the crude extracts of strain PA19 treated medium was 2340 cm^{-2} (Figure 2).

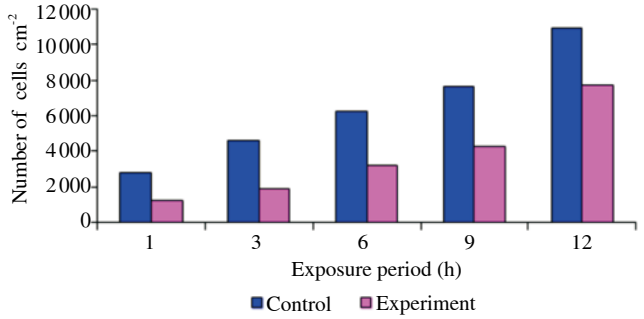

Figure 1. Adhesion assay using biofilm-forming bacterial strain *Alteromonas* sp. treated with the crude extracts of strain PA8 (*Exiguobacterium* sp.). Control: without crude extract treatment.

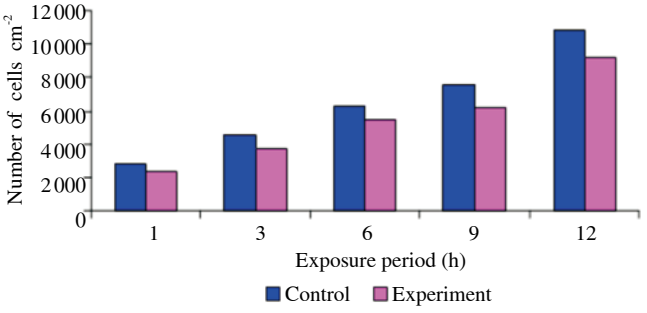

Figure 2. Adhesion assay using biofilm-forming bacterial strain *Alteromonas* sp. treated with the crude extracts of the strain PA19 (*Actinobacterium* sp.). Control: without crude extract treatment.

After 12 h of glass surface immersion, the number of *Alteromonas* sp. cells found on the coupons submerged in medium treated by strain PA8 was 7676 cm^{-2}, while on the coupons kept in control medium, 10872 cells cm^{-2} were adhered on the glass surface. The glass coupons submerged in medium treated by strain PA19 showed a density of 9231 cm^{-2} *Alteromonas* sp. cells at the end of the experiment (after 12 h). On the coupons kept in the control medium, 10 872 cells cm^{-2} cells were observed during the same period. Two-way ANOVA showed a significant variation on the adhesion of bacteria between the glass coupons submerged in control medium and medium treated by strain PA8 extracts (Table 4). Also, bacterial adhesion showed a significant difference between the control and strain PA19 extract treatment (Table 4).

3.5. TLC analysis

The crude extracts of the surface-associated bacterial strains PA8 and PA19 were analyzed by TLC. The R_f value of the compound present in the crude extracts isolated from the strain PA8 was 0.72 cm. The crude extracts of the strain PA19 showed a single spot with the R_f value of 0.53 cm (Figure 3). The compound resolved from the strain PA8 showed inhibitory activity against all the four target bacteria. The diameter of zone of inhibition against *Gallionella* sp. was 10 mm and 15 mm against *Alteromonas* sp. It also showed an inhibition zone of 15 mm against *Klebsiella* sp. The bioactive compound recovered from the strain PA19 showed an inhibition zone of 6 mm against *Alteromonas* sp. and 7 mm against *Gallionella* sp.

Table 4

Two-way ANOVA (analysis of variance) of adhesion of *Alteromonas* sp. treated with crude extracts of bacterial strains (strains PA8, PA19) associated with polychaetes. Adhesion assay duration (time) and treatments (extract-treated and control) were considered as factors.

Source of Variation	PA8 extracts				PA19 extracts			
	SS	*df*	*MS*	*F*	*SS*	*df*	*MS*	*F*
Time	62173245	4	15543311	64.47*	64177205	4	16044301	131.33*
Treatment	19171172	1	19171172	79.52*	2751003	1	2751003	22.51*
Error	964287.4	4	241071.9		488653	4	122163.3	
Total	82308704	9			67416860	9		

*$P < 0.05$.

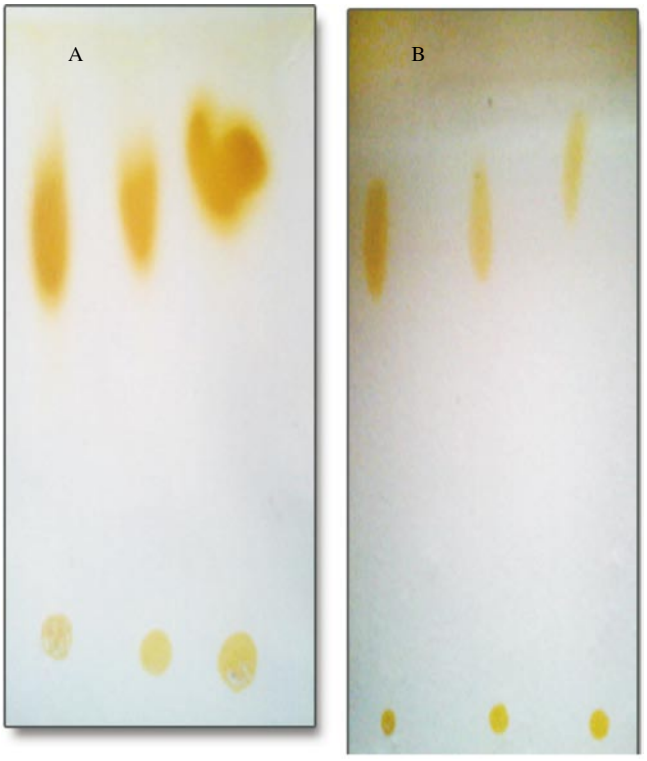

Figure 3. Thin-layer chromatography analysis of crude extracts of bacterial strains associated with polychaetes.
A: Strain PA8; B: Strain PA19. Replicate (n = 3) samples were loaded on glass plates coated by silica gel while *n*-butanol, acetone and water (4:5:1) were used as solvents.

3.6. Phylogenetic analysis of bacterial strains associated with polycahetes

The bacterial strains PA8 and PA19 were identified by sequencing the 16S rRNA gene. The bacterial strain PA8 isolated from the surface of *Platynereis dumerilii* was closely related to *Exiguobacterium lactogenesis* (99% similarity) available in the NCBI database and identified as *Exiguobacterium* sp. CMST SSS-3 (GenBank:

HM851458.1). The strain PA19 isolated from the surface of the polychaete *Syllis variegata* was identified as *Actinobacterium* CMST SSS-4 (GenBank: HM851459.1). It showed 99% similarity with the *Actinobacterium* C19 gene in the Genbank database.

4. Discussion

Polychaetes are the major benthic communities in the marine ecosystems in terms of diversity and abundance. Investigations on the bacteria associated with polychaetes are limited when compared to other marine organisms[28,29]. In the present study, of the total 32 bacterial strains isolated from two polychaete species, 13 strains showed antimicrobial activity. The greater number of cultivable bacteria with antimicrobial activity isolated from polychaetes indicates the significance of this group for marine microbial bioactive compounds discovery. Phylogenetic analysis (based on 16S rRNA) of the two bacterial strains associated with polychaetes has expanded our knowledge on the association of microorganisms with marine organisms.

Previous studies reported the presence of various bacterial genera from the surface of marine invertebrates[30-33]. In the present study, two bacterial strains associated with the polychaetes were identified as *Exiguobacterium* sp. and *Actinobacterium* sp. The crude methanolic extracts of these two strains showed strong antibacterial and antibiofilm activities in different assays. *Exiguobacterium* is Gram-positive and facultative anaerobe belonging to the class of Bacilli and can be isolated from various sources including marine environment. Pathak *et al*.[34] reported the antimicrobial properties of *Exiguobacterium* sp. HKG-126 isolated from the mangrove region of west coast in India. Previous studies also reported the association of *Exiguobacterium* with marine organisms such as bryozoans[35], ascidians[36] and from marine sediments[37]. In a previous study, Rajasree *et al*.[38] reported the biofilm inhibitory activity of extracellular polymeric substances produced by the strain *Exiguobacterium* sp. CMST SSS-3.

Actinobacterium, a Gram-positive filamentous bacteria, belongs to Actinomycetes group and widely distributed in natural and man-made environments. Actinobacteria are the important bacterial group due to their biotechnological significance as they are reported to produce bioactive metabolites which include antibiotics, antitumour agents, cosmetics and pesticides[39,40]. Many new chemical molecules and bioactive metabolites were also reported from marine Actinobacteria[5]. Actinobacteria and marine invertebrate association are mainly documented for sponges in the literature[4,5,41,42] and relatively little information is available on other macroorganisms.

In addition to traditional antimicrobial screening for possible drug molecules, we conducted different assays to understand the biofilm inhibitory activities of bacteria associated with polychaetes. Adhesion of microbes on hard surfaces submerged in any environment including human body is leading to the formation of biofilms. Generally, biofouling in aquatic environment begins with the development of biofilm and bacteria are considered to be the major organisms in the biofilms[43,44]. Hence, the adhesion assay using bacteria isolated from the biofilms may be useful for the formulation of natural-product-based biofilm inhibitory and antifouling agents.

Adhesion and assay showed that the crude extracts of strain PA8 (*Exiguobacterium* sp.) and PA19 (*Actinobacterium* sp.) inhibited the settlement of biofilm-forming bacteria on glass surfaces considerably. These findings confirms a growing number of previous studies which support the antagonistic activities of marine bacteria associated with surfaces[2,23,45]. In the present study, a simple adhesion assay has been designed in order to quantify the attachment of bacteria on glass surface. Though this method has limitations on the enumeration of bacterial population with microscope compared to microtitre plate assays, it gives results with a short period and the replication used in the present study is significant enough to prove that adhesion of bacterial cells varied considerably in control group and experiemental group treated by surface-associated bacterial extracts.

The BATH assay[27], also called as microbe adherence to hydrocarbons assay, showed that the surface-associated bacterial extracts affected cell surface hydrophobicity of the biofilm-forming bacteria. The compounds which affect the cell surface properties such as hydrophobicity, surface energy, *etc*. may inhibit or minimize the adhesion of bacterial cells on surfaces. The bacterial cell hydrophobicity is influenced by factors such as the presence of cell appendages containing proteins[46-48] and extracellular polymeric substances[49,50]. Generally, all fouling organisms including bacteria use sticky material for the adhesion to surface[51]. Extracellular polymeric substances produced by the bacteria during attachment on surface are one of the important adhesives involved in the biofilm formation. The EPS helps the microbes to attach on surfaces and form biofilm and the EPS is likely to play a crucial role in minimizing the repulsive force between microorganisms and surface and cell-to-cell adhesion[52].

The reduction in the concentration of EPS (carbohydrate and protein content) produced by the biofilm-forming bacterial strains when treated with surface-associated bacterial extracts observed in this study indicates the alteration of physiology of the biofilm-forming bacteria. However, this assumption needs further confirmation by detailed studies on structural and physiological changes in bacterial cells when treated with biocides. In a previous study by Rathi and Satheesh[53], it was reported that treatment of biofilm-forming bacterial cells with sodium hypochlorite (used for removal of biofilms) affected the cell surface hydrophobicity and EPS production and thereby reduced the adhesion. Results obtained in this study also showed the same effects of chlorine, a common agent used to control biofilms and biofouling in aquatic environments.

As for the effectiveness of the methods applied to extract the bioactive fraction from the bacteria, some studies showed that methanol extraction yielded higher antimicrobial activity than *n*-hexane and ethyl acetate extraction[54]. However, another study by Sastry and Rao preferred chloroform than methanol and benzene[55]. It is clear that different organic solvents are used for extracting bioactive compounds depending on the nature of the organisms and objectives of the study. In the present study, only methanol was used for crude extract preparation from surface-associated bacteria based on the previous experience in the screening of antimicrobial compounds by Delval *et al*.[56]. Also, our aim was to observe the antagonistic activity of the bacteria associated with polychaetes rather than isolation and characterization of bioactive molecules. The development of compounds from marine organisms for pharmaceutical and other applications has limitations due to the problem in getting the supply of organisms and associated biodiversity concern. Associations between marine organisms and microbes are observed

all over the marine ecosystems and number of such marine microbes are documented in the literature[57]. The production of compounds by microbes that are found on the surface of organisms would be extremely advantageous as many of these microbes are less difficult to culture[58] so that the bottleneck problem of marine natural product supply can be solved[57].

In conclusion, the present study indicates that bacteria associated with marine invertebrates could be used as a potential source for searching bioactive molecules, drugs and antifouling compounds. Since only a minor proportion of microorganisms associated with marine organisms has been screened for the production of bioactive compounds, it is necessary to focus more research to utilize their bioactive potential. Besides, the present study confirms that as a dominant groups in the benthic ecosystems, polychaetes harbour abundant microbial populations on their surfaces like other marine organisms such as sponges, ascidians and seaweeds. Further, culture-independent studies on diversity of microbes associated with polychaetes will reveal more bacterial groups and improve their application in biotechnology.

Conflict of interest statement

We declare that we have no conflict of interest.

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