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Arctic Actinomycetes as Potential Inhibitors of *Vibrio cholerae* Biofilm

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Abstract The aim of this study was to identify novel biofilm inhibitors from actinomycetes isolated from the Arctic against *Vibrio cholerae,* the causative agent of cholera. The biofilm inhibitory activity of actinomycetes was assessed using biofilm assay and was confirmed using air–liquid interphase coverslip assay. The potential isolates were identified using 16S rRNA gene sequencing. Of all, three isolates showed significant biofilm inhibition against *V. cholerae*. The results showed that 20% of the actinomycetes culture supernatant could inhibit up to 80% of the biofilm formation. When different extracted fractions were assessed, significant biofilm inhibition activity was only seen in the diethyl ether fraction of A745. At 200 μg ml^{-1} of diethyl ether fraction, 60% inhibition of *V. cholerae* biofilm was observed. The two potential isolates were found to be *Streptomyces* sp. and one isolate belonged to *Nocardiopsis* sp. This is the first report showing a *Streptomyces* sp. and *Nocardiopsis* sp. isolated from the Arctic having a biofilm inhibitory activity against *V. cholerae.* The spread of drug resistant *V. cholerae* strains is a major clinical problem and the ineffectiveness in antibiotic treatment necessitates finding new modes of prevention and containment of the disease, cholera. The formation of biofilms during the proliferation of *V. cholerae* is linked to its pathogenesis. Hence, the bioactive compound from the culture supernatant of the isolates identified in this study may be a promising source for the development of a potential quorum sensing inhibitors against *V. cholerae*.

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Introduction

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The worldwide spread of multidrug resistant bacteria is an alarming issue, as it renders antibiotics ineffective in treating infections. Hence, novel strategies need to be found to control bacterial diseases. An alternative to the present conventional methods is specific attenuation of bacterial virulence, which targets certain regulatory processes that control the virulence factors. Quorum sensing (QS) is the regulatory system that controls phenotypic characters such as biofilm formation, toxin production, swarming, and bioluminescence. Inhibition of QS has been proven to be a very potent method for inhibition of bacterial virulence. The biofilm structures exhibit a higher tolerance to antimicrobial agents [6]. *Vibrio cholerae* is a natural inhabitant of aquatic ecosystems. The organism's ability to cause periodic outbreaks can be linked to its survival mechanisms in various aquatic habitats. *V. cholerae* can attach to a variety of surfaces under different environmental conditions. Thus, its attachment to surfaces and subsequent biofilm-mediated growth mode facilitates the persistence and survival of *V. cholerae* [3].

Yang et al. have shown that *V. cholerae* enters the host mainly in the form of biofilms. The number of cells within biofilm is sufficient enough to induce symptomatic cholera [17]. The viable cells in biofilms are resistant to getting

killed by acid shock as compared with planktonic cells and this resistance enhances its survival during its passage through the gastric environment. It was found that 40% of the cell wall proteins of cells from biofilms are different from planktonic cells [11, 14]. Many reports have showed that biofilm formation in *V. cholerae* is controlled by quorum sensing [5, 12, 13]. Quorum sensing and biofilm formation are also involved in transition of *V. cholerae* to a conditionally viable environmental *V. cholerae* (CVEC) form, which are resistant to cultivation, using standard methods. However, they revive into culturable and virulent forms when inoculated into the ileal loops of adult rabbits [4]. The basic treatment for cholera patients is rehydration and antibiotics [15]. As multidrug resistance in *V. cholerae* is increasing, there is an urgent need for novel compounds which will interfere with their quorum sensing.

The Arctic has been geographically isolated for millions of years and harbors some of the most unexplored and unexploited bioresources on Earth. Hence, this unique ecosystem can serve as a potential region for obtaining novel bacteria with therapeutic applications. Hence, the present study is an attempt to screen and identify potential actinomycetes from the Arctic, inhibiting biofilms of *V. cholerae*.

Materials and Methods

Sampling Site

The sediment samples pertaining to this study were collected using a Grab sampler, from four different regions of Kongsfjorden system, Ny-Alesund, an island in the Svalbard Archipelago (79°55′N, 11°56′E), during the Indian Arctic Expedition-2009, organized by the Ministry of Earth Science, Govt. of India.

Isolation of Actinomycetes

The collected sediment samples were transported in dry ice to the laboratory in India. For isolation of Arctic actinomycetes, the sediment samples were serially diluted in sterile distill water and plated on to starch casein agar and actinomycetes isolation agar plates and incubated at 18°C for 3–5 days. The isolated colonies were stabbed into half-strength starch casein media and were stored for further analysis.

Biofilm Assay

Vibrio cholerae O1 (MCV09) was used as test strain for biofilm inhibition assay. The actinomycete isolates were inoculated into starch casein broth and incubated at 28°C for 3–7 days. After incubation, the cultures were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was then transferred into another sterile screw cap tube under aseptic conditions and stored at −20°C for further use. The biofilm assay was carried out as described previously [9]. Briefly, 10 µl of overnight culture of *V. cholerae* was inoculated in 96-well microtitre plates (polystyrene) containing Luria-Bertani (LB) broth and different percentage of actinomycetes culture supernatants (10, 20, 30, 50% v/v) followed by incubation for 48 h at room temperature. Planktonic cells and spent media were discarded, and adherent cells were gently rinsed twice with deionised water and allowed to air dry before being stained. The biofilms were stained with 210 µl 0.1% crystal violet solution (w/v) for 10 min, after which the dye was discarded and the wells were rinsed twice with deionized water. The wells were allowed to air dry before solubilization of the crystal violet with 210 µl of dimethyl sulfoxide (DMSO). The optical density was determined at 595 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad). The biofilm inhibition of *V. cholerae* in borosilicate glass tubes was also examined.

Air–Liquid Interphase Coverslip Assay

Vibrio cholerae was incubated in a 50 ml Erlenmeyer flask containing 6 ml LB medium with a cover glass at 28°C for 48 h. To evaluate the inhibition of the biofilm, the biofilm was also treated with extracts of selected actinomycete strains. After incubation, the cover slips were taken out and washed with deionised water. The cover slips were fixed with 2% glutaraldehyde solution and stained with 0.1% acridine orange for 10 min. Later the cover slips were washed with water, air dried and mounted on a clean glass slide and observed under inverted fluorescent microscope with 63× oil immersion objective (Nikon).

Molecular Identification of Actinomycetes

Total genomic DNA of selected *Actinomycete* isolates (A745, A733 and A731) was isolated using commercially available fungal genomic DNA isolation kit (Chromous Biotech). The identification of the test strains was done by sequencing the conserved 16S rRNA gene as described previously [16].

Extraction of the Bioactive Compound

The potential isolate A745 was inoculated into 500 ml of starch casein broth and incubated at 28°C on a rotary shaker for 5 days. After incubation period, the culture was centrifuged at 12,000 rpm for 5 min at 4°C. Bioactive principle containing supernatant was extracted using equal

volume of different organic solvents such as hexane, diethyl ether, ethyl acetate and butanol based on their increasing polarity. The solvent extracts thus obtained were concentrated using a rotary evaporator (Rotavapor RII, Buchi, Switzerland). The crude extracts were dissolved in dimethyl sulphoxide, so as to prepare a stock of 100 mg ml^{-1}. Biofilm inhibition assay was done with different concentrations of this crude extract.

Results

Thirty-one actinomycetes were isolated from the four sediment samples collected from the Kongsfjorden system. Of these, three isolates (A731, A733, and A745) showed significant reduction in biofilm formation of *V. cholerae* (Fig. 1). Among the three isolates, A745 showed the maximum inhibition, followed by A733 and A731. Results showed that 20% supernatant of A745 induced ~88% inhibition in the biofilm formation, while the other two showed 80% inhibition (Fig. 2). There was no further inhibition in any of the isolates when the percentage of the culture supernatant was further increased. The results obtained from the biofilm assay were further confirmed by air–liquid interphase coverslip assay. The coverslip assay results also showed the reduction in adhesion of the cells onto the coverslip surface confirming the biofilm inhibitory activity of the extracts (Fig. 3). The biofilm inhibitory activity of the isolates could be due to the reactivity of the bioactive compound present in the culture supernatant with secreted molecules such as exopolysaccharides or other adhesins. Results obtained from the biofilm assay and air–liquid interphase coverslip assay confirm that culture supernatants have a significant inhibitory activity on the biofilm formation without inhibiting the growth of the bacteria. The two isolates (A733 and A745) were identified as *Streptomyces* sp. and isolate (A731) as *Nocardiopsis* sp. based on 16S rRNA gene sequence and BLAST analysis

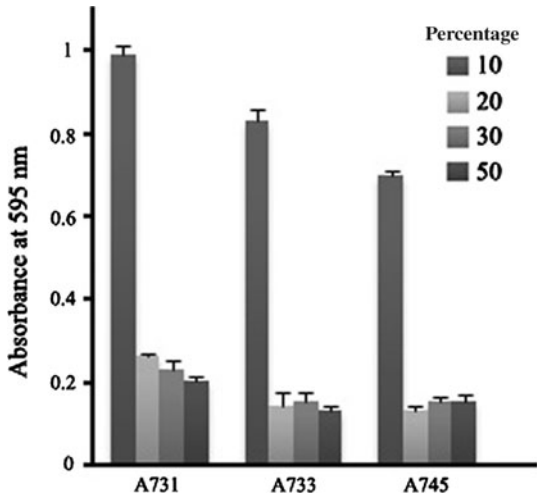

Fig. 2 Graph showing reduction in *V. cholerae* biofilm formation with different percentages of actinomycetes supernatant

(Accession No: JF903931, JF903932 and JF903933, respectively). As A745 showed maximum biofilm inhibition, its culture supernatant was further subjected to solvent extraction for the purification of the bioactive molecule. Of the various fractions tested, significant biofilm inhibition activity was only seen in diethyl ether fraction. At 200 µg ml^{-1} of diethyl ether fraction, 60% inhibition of *V. cholerae* biofilm was noticed. Increase in its concentration showed an ascending trend in the biofilm inhibition activity (Fig. 4).

Discussion

The rapid emergence of microbes that resist most commonly used and even newly developed antibiotics has emphasized the need for the development of new strategy against infectious diseases. As similar to many opportunistic pathogens, *V. cholerae* also rely on QS, a bacterial

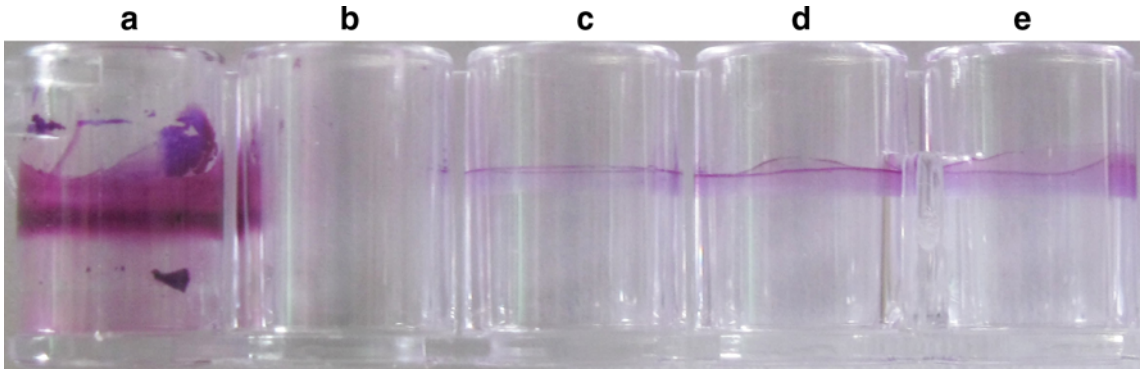

Fig. 1 Biofilm inhibition assay. **a** *V. cholerae* biofilm (positive control); **b** LB broth (negative control); **c**, **d**, **e** biofilm treated with 20% supernatant of isolates A731, A733 and A745

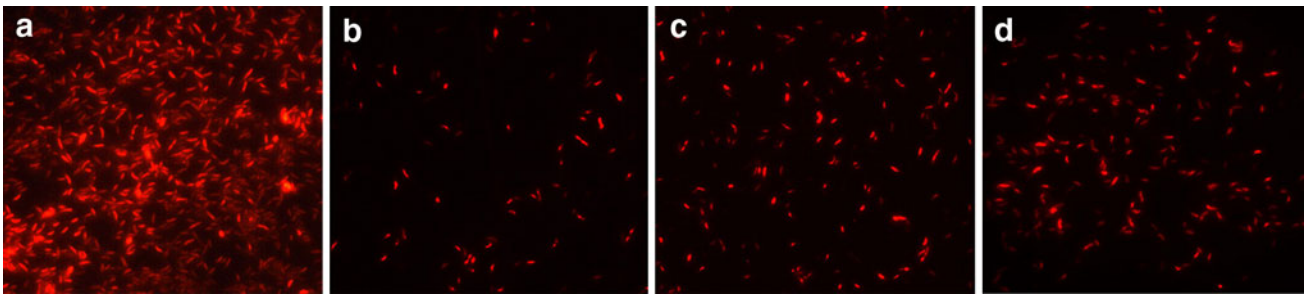

Fig. 3 Air–liquid interphase coverslip assay. **a** *V. cholerae* biofilm (positive control); **b**, **c**, **d** biofilm treated with supernatant of isolates A731, A733 and A745

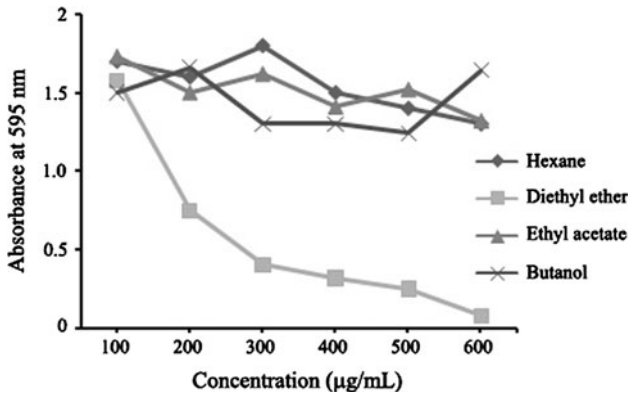

Fig. 4 Graph showing biofilm inhibition of *V. cholerae* by different concentrations of diethyl ether crude extract of A745

cell-to-cell communication system for biofilm formation and virulence character expression. The QS is crucial for execution of pathogenesis called quorum quenching or anti-pathogenic approach which will be the promising alternative strategy in future to control infectious diseases. As QS does not unswervingly involve in the elementary process of bacterial growth, inhibition of QS does not enforce harsh selective pressure which brings forth resistance.

There are reports of various plant extracts, algae, bacteria and *N*-acyl homoserine lactone (AHL) degrading enzymes having biofilm inhibitory activity against various pathogenic microorganisms [7]. Our group has already reported the biofilm inhibition property of AiiA enzyme from *Bacillus* sp. against *V. cholerae* [1] and the ability of these enzymes to interfere with the virulence attenuation in *Erwinia carotovora* has been reported by other workers [8]. Homologues of AiiA enzyme are also reported in other microorganisms like *Agrobacterium tumefaciens, Rhodococcus* sp., and *Arthrobacter* sp. [10]. These prokaryote to prokaryote and eukaryote to prokaryote quorum quenching mechanisms available in nature may be the strategies to compete with other microbes in nature. In the present investigation, actinomycetes isolated from the Arctic were checked for biofilm inhibitory activity and from the results it was revealed that the isolates A745, A733 and A731 showed significant anti-biofilm activity. This is the first report of Arctic *Streptomyces* and *Nocardiopsis* sp. showing significant biofilm inhibitory activity against *V. cholerae*. Moreover, this is also the first report showing a *Nocardiopsis* sp. with biofilm inhibitory activity against *V. cholerae*.

Biofilms are involved in most of the bacterial infections in humans and as no specific biofilm inhibitors are yet available commercially, there is an urgent need for exploring novel bioactive molecules. As biofilm formation is a quorum sensing dependent process in *V. cholerae*, the bioactive compound present in the diethyl ether fraction of the isolate A745 identified in this study may be a promising source for the development of a potential quorum sensing inhibitor against *V. cholerae*. According to the available reports, *Streptomyces* spp. alone has contributed to 60–70% of the bioactive molecules discovered till date [2]. For the first time, a *Nocardiopsis* sp. has been reported, through this study, with biofilm inhibitory activity and has shown that rare actinomycetes have also gained importance in order to enhance the rate of discovery of novel and potent therapeutic agents. Further studies in our laboratory are in progress in the identification of bioactive compounds and the determination of molecular mechanisms involved in quorum quenching and *V. cholerae* pathogenesis.

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References

1. Augustine N, Kumar P, Thomas S (2010) Inhibition of *Vibrio cholerae* biofilm by AiiA enzyme produced from *Bacillus* spp. Arch Microbiol 192:1019–1022

2. Ceylan O, Okmen G, Ugur A (2008) Isolation of soil Streptomyces as source antibiotics active against antibiotic-resistant bacteria. EurAsia J BioSci 2:73–83
3. Ghannoum MA, O'Toole GA (2004) Microbial biofilms. Amer Society for Microbiology, Washington, DC, pp 320–321
4. Kamruzzaman M, Udden SM, Cameron DE, Calderwood SB, Nair GB, Mekalanos JJ, Faruque SM (2010) Quorum-regulated biofilms enhance the development of conditionally viable, environmental *Vibrio cholerae*. Proc Natl Acad Sci USA 107: 1588–1593
5. Kelly RC, Bolitho ME, Higgins DA, Lu W, Ng WL, Jeffrey PD, Rabinowitz JD, Semmelhack MF, Hughson FM, Bassler BL (2009) The *Vibrio cholerae* quorum-sensing autoinducer CAI-1: analysis of the biosynthetic enzyme CqsA. Nat Chem Biol 5:891–895
6. Kierek K, Watnick PI (2003) Environmental determinants of *Vibrio cholerae* biofilm development. Appl Environ Microbiol 69:5079–5088
7. Kociolek MG (2009) Quorum-sensing inhibitors and biofilms. Anti-Infect Agents Med Chem 8:315–326
8. Pan J, Huang T, Yao F, Huang Z, Powell CA, Qiu S, Guan X (2008) Expression and characterization of *aiiA* gene from *Bacillus subtilis* BS-1. Microbiol Res 163:711–716
9. Pratt LA, Kolter R (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 30:285–293
10. Rasmussen TB, Givskov M (2006) Quorum-sensing inhibitors as anti-pathogenic drugs. Int J Med Microbiol 296:149–161
11. Ren D, Zuo R, Gonzalez Barrios AF, Bedzyk LA, Eldridge GR, Pasmore ME, Wood TK (2005) Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. Appl Environ Microbiol 71:4022–4034
12. Rutherford ST, van Kessel JC, Shao Y, Bassler BL (2011) AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. Genes Dev 25:397–408
13. Sultan SZ, Silva AJ, Benitez JA (2010) The PhoB regulatory system modulates biofilm formation and stress response in El Tor biotype *Vibrio cholerae*. FEMS Microbiol Lett 302:22–31
14. Tamayo R, Patimalla B, Camilli (2010) A Growth in a biofilm induces a hyperinfectious phenotype in *Vibrio cholerae*. Infect Immun 78:3560–3569
15. Van Dellen KL, Watnick PI (2006) The *Vibrio cholerae* biofilm: a target for novel therapies to prevent and treat cholera. Drug Discov Today Dis Mech 3:261–266
16. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697
17. Yang M, Frey EM, Liu Z, Bishar R, Zhu J (2010) The virulence transcriptional activator AphA enhances biofilm formation by *Vibrio cholerae* by activating expression of the biofilm regulator VpsT. Infect Immun 78:697–703