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Reconfiguring the quorum sensing regulator SmcR of *Vibrio vulnificus* to control virulence via endophytic fungal compound-Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)

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ABSTRACT

Vibrio vulnificus is a mortiferous pathogen accounting for 95% of all deaths related to sea foods. The objective of this study is to isolate secondary metabolites from endophytic fungi, that binds to *SmcR* (a quorum sensing regulator) in such a way that it down regulates metallo protease gene with no alterations made in the domain of *SmcR* that represses hemolysin. The extract was analyzed by GC-MS, from which seven secondary metabolites were identiûed by comparing the mass spectral data with NIST library. Potential lead (VVSI3) was found through docking studies that could effectively bind to *SmcR* and curb its action. Based on this, *in vitro* work was carried out which exhibited a noteworthy drop-off in protease activity of *Vibrio vulnificus*, proving it to be a potential drug lead.

KEYWORDS

Vibrio vulnificus;
Heomolysin;
Protease;
Aspergillus terreus.

INTRODUCTION

Vibrio vulnificus, a human opportunistic pathogen causes gastrointestinal infections leading to primary sepsis and wound infection[1,2]. Based on the biochemical, serological properties and host specificity, *Vibrio vulnificus* is classified into biotype 1, 2 and 3[3-6]. Biotype 1 causes disease in human after ingestion of raw oysters or through open wound infection through infected seawater. On the other hand biotype 2 strains are found only in eels or seawater inhabited by eels under normal circumstances but infects humans when diseased eels are consumed[7,8]. Later biotype 3, a hybrid of biotype 1, 2 was identified in fresh water farms, which also causes large disfiguring ulcers that require extensive debridement or even amputation. But among all the biotypes, biotype 1 is common causing infection along the coast of several countries like USA, Korea, Europe, Taiwan, China and commonly seen in Gulf of Mexico[9-13]. *Vibrio vulnificus* illness has one of the highest mortality rates of any foodborne disease and has emerged as a food safety issue in a number of countries. Very few studies were only carried out on the occurrence of *Vibrio vulnificus* in tropical waters such as India. Hence very little information is available on the prevalence and habitat of this organism in tropical

waters. The pathogenicity of these strains are controlled by the quorum sensing system involving a quorum regulator, SmcR. It imparts a strong control over cytotoxicity by parallel y repressing hlyU, an activator of rtxA1 and vvhA[14].

Mutualism is observed between endophytic fungi and many plants in which the fungi colonize the plant tissue without causing evident harm to them, in turn protecting them from the invasion of pathogens. These wide ranges of endophytic fungi produce antimicrobials that have shown to aid the battle against pathogens and even cancers in animals and humans[15,16].

So we aim to screen, identify an endophytic fungal compound to antagonize SmcR action over cytotoxicity and retaining its same behavior of repressing hlyU.

MATERIALS AND METHODS

Test strain used for the study

The test microbial strain of human pathogen, *Vibrio vulnificus* [MTCC 1145] was procured from the Institute of Microbial Technology [IMTECH].

Sample collection, processing and identification of an endophytic fungi

The mangrove leaves collected from the coast of Chidambaram, Cuddalore District, Tamil Nadu were filtered to remove the suspended sand and other dirt particles. Further they were surface sterilized in 5% Sodium hypochlorite for 5 minutes followed by treating with 75% ethanol for 30 seconds. The surface sterilized leaves were chopped, grounded in a mortar and pestle and added onto 100ml of Potato Dextrose Broth (PDB) and incubated at 37°C. As the fungal growth was observed, subsequent serial dilution was done to obtain pure culture of endophytic fungi in Potato Dextrose Agar (PDA). The fungal species were identified targeting their 16srRNA genes.

Secondary metabolites extraction and fractionation

The identified endophytic fungi, *Aspergillus terreus* were grown in PDB in static condition at 37°C for 30 days. The extract was separated using filtration procedure [Whatman No 1]. Ethyl acetate was added in culture filtrate and the compounds were separated using a separating flask and concentrated in a rotary vacuum evaporator. The dry semi solid residue was again dissolved in ethyl acetate and 1 µl of the sample was injected to GC-MS analysis (Perkin Elmer Clarus -500) to identify its constituent compounds. The conditions were set as follows: the oven temperature was set with a range of 50°C at 8°/min to 220°C [2 min] at 7°C/min to 280°C, the mass range was set at 40 to 600amu and the electron ionization was followed. The compounds were identified in comparison with the obtained spectra with the NIST 2005(National Institute Standard and Technology) mass spectral library.

Ligand and protein preparation

A total of seven compounds were obtained by the GC-MS analysis of the ethyl acetate extract of (EF). The molecular model of SmcR was prepared using Schrödinger's protein preparation wizard and a rigid model was prepared. The ligand structures were downloaded from Pubchem [http://pubchem.ncbi.nlm.nih.gov/] in SDF format and were prepared for docking using Schrödinger's ligprep module. Dimerization is found to be important for the SmcR-DNA complex stabilization. It has been already found that the sites Leu-165, Tyr-171, Tyr-193, and Met-196 are critical for dimerization in SmcR and hence they were chosen as the target sites.

Docking studies

Docking studies were carried out using glide module of Schrödinger. The ligands that docked with low binding energy producing high glide scores were taken to potential leads.

In vitro experiments to assess anti-quorum effect

To study the quorum effect of *Vibrio vulnificus*, experimental studies (growth curve, total protein, protease assay, hemolysin assay) were done by growing *Vibrio vulnificus* in nutrient broth with 2% NaCl containing varying concentration of the extract (1, 2, 3, 4, 5, 10µg/ml) respectively. The dose response effect of the crude library of endophytic compound was observed in a time dependent manner (0, 2, 4, 12 hours).

1) Growth curve study

The sample which was collected with a time interval of two hours was measured for absorbance at 600nm

to check for antimicrobial activity of the extract which leads to reduction in O.D.

2) Total protein estimation

The total protein was estimated by Hartree-Lowry's method. To 1mL of sample, 0.9mL of reagent A (7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration) was added and was incubated for ten minutes in water bath (50 ^{0}C). After the mixture was cooled to room temperature, 0.1mL of reagent B (70 mM Na-K tartrate, 40 mM copper sulfate) was added and incubated at room temperature for 10 minutes. 3mL of reagent C (1 volume Folin-Ciocalteau reagent diluted with 15 volumes of water) was added quickly to the above mixture and incubated for ten minutes in a 50 ^{0}C bath. The absorbance was read at 655nm[17].

3) Protease assay

Azocasein was the substrate used to elucidate protease activity. 100µl of the sample supernatant was taken along with 400µl of 1% azocasein and kept at 37 C, undisturbed for 30 minutes. Then 600µl of 10% trichloric acid was added and incubated on ice for 30 minutes. After 30 minutes the mixture was centrifuged and supernatant was recovered. To 800µl of supernatant 200µl of 1.8N sodium hydroxide was added. Then absorbance was measured on a spectrophotometer at 420 nm[18].

4) Hemolysin assay

50µl of 3.5% of sheep blood in Phosphate buffered saline (PBS) was added to 450µl of sample supernatant and kept at 30C for one hour. Then this mixture was centrifuged and its absorbance was measured with a spectrophotometer at 405nm. The experiment was performed in triplicates. The percentage of hemolysis was calculated by finding the difference in absorbance of the sample and blank divided by the absorbance of the control (maximum absorbance). The control was 100% osmotic hemolyzed sheep erythrocytes in de-ionized water.

RESULTS

NCBI – BLAST results showed that the endophytic fungus was *Aspergillus terreus.*

GC-MS analysis of ethyl acetate extract of Endophytic fungi: The ethyl acetate extract of EF was sent for GC/MS analysis and total of 7 secondary metabolites were identiûed by comparing the mass spectral data with NIST library (TABLE 1).

TABLE 1: List of ligands identiûed from (EF) using GC-MS analysis

S.No.	Peak Name	Retention time
1	Name: Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)- Formula: $C_{15}H_{24}$, MW: 204	15.42
2	Name: Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]- Formula: $C_{15}H_{24}$, MW: 204	16.05
3	Name: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- Formula: $C_{11}H_{18}N_2O_2$, MW: 210	22.14
4	Name: Sperminutese Formula: $C_{10}H_{26}N_4$, MW: 202	22.65
5	Name: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- Formula: $C_{11}H_{18}N_2O_2$, MW: 210	23.73
6	Name: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- Formula: $C_{11}H_{18}N_2O_2$, MW: 210	24.07
7	Name: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- Formula: $C_{14}H_{16}N_2O_2$, MW: 244	33.40

Docking studies

Docking analysis was carried out using Schrodinger software to find potential leads that would effectively bind to SmcR and curb its action. The structure of SmcR was referred from Kim *et al.*[19]. The compounds that showed promising results are shown in the Figure 1. It was found that VVSI3 showed the best interaction with good binding energy (-5.71 kcal/mol). The other compound VVSI7 had a binding energy of -5.5 kcal/mol (Figure 2). By comparing the results it was found that Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) showed potential to be used as a promising lead after further structural modifications (Figure 3).

In vitro assay results for regulation of the expression of the extracellular virulence factors encoded by

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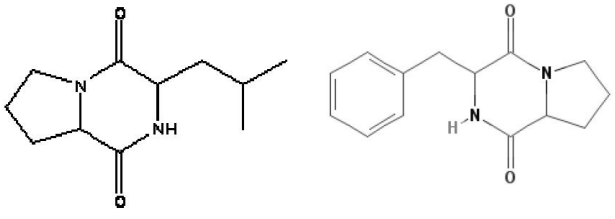

(A) (B)

Figure 1 : The ligands that showed best binding energy with SmcR identified from ethyl acetate extract of (EF): A- Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) VVSI3; B- Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) VVSI7

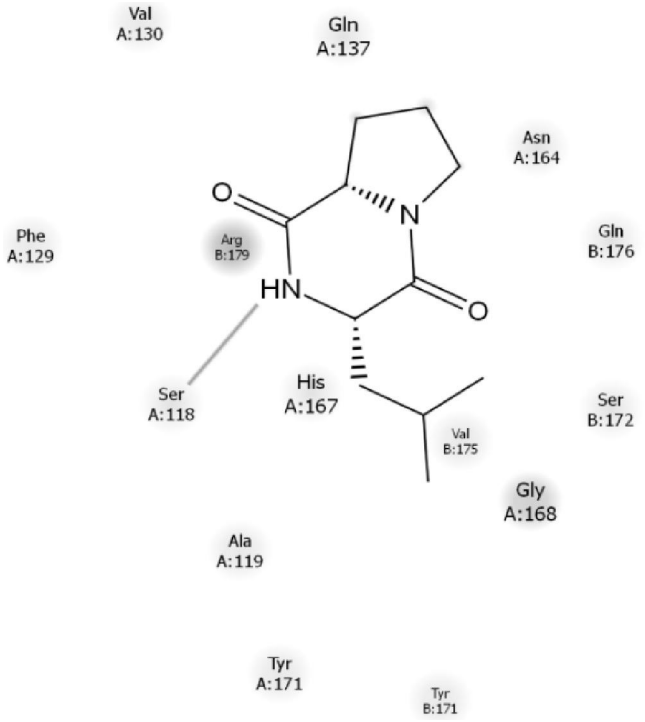

Figure 2 : VVSI3 making hydrogen bond with the Ser 118 of SmcR. Residues within 4A^0 are also shown

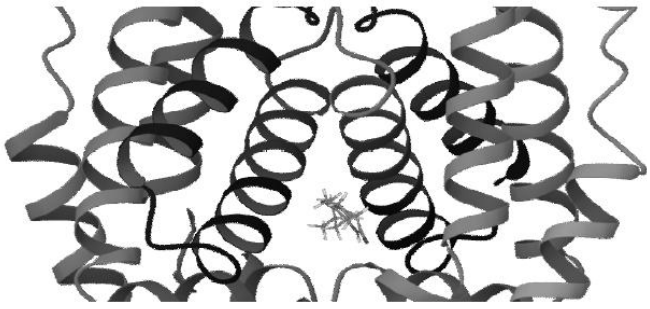

Figure 3 : Docked model of ligand(VVSI3) into the active site of SmcR

vvpE and *vvhA* - The expression levels of *vvpE* and *vvhA* were analysed under various phases of anti SmcR Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) in a time dependent manner in *Vibrio vulnificus* has been analyzed (Figure 4). The fungal endophytic crude extract has been tested against the growth of *Vibrio vulnificus*. Growth seem to be unaffected even after the addition of the endophytic crude extract fungal extract increases growth seems to be considerably affected. This reveals that the endophytic fungal extract does not inhibit growth of *Vibrio vulnificus* which shows that it may work through quorum sensing pathway.

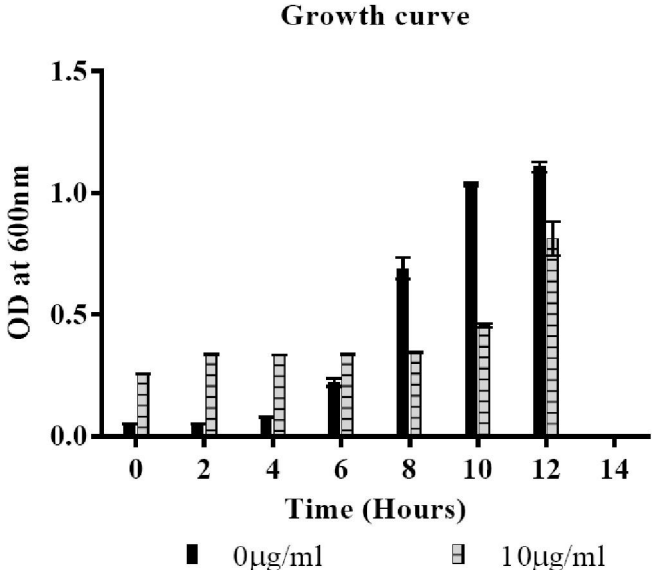

Figure 4 : Time and concentration dependent analysis of the cell growth

Proteolysis was found to be rapid between the hours 6 to 8 (Figure 5). Hence variable concentration of endophytic fungal extract was tested against the proteolysis for that specific time period. Considerable decrease in proteolysis was observed in all concentrations of the crude extract. Hemolysis activity was observed as depicted in Figure 6.

The first graph is the positive control (without the extract) and the second shows the % Hemolytic activity at 10µg/ml. It could be inferred from the graph that the % Hemolytic activity remains repressed after addition of the Endophytic fungal extract.

DISCUSSION

The objective in view was to regulate the quorum sensing receptor of *Vibrio vulnificus* in a way that it can down regulate protease without affecting the do-

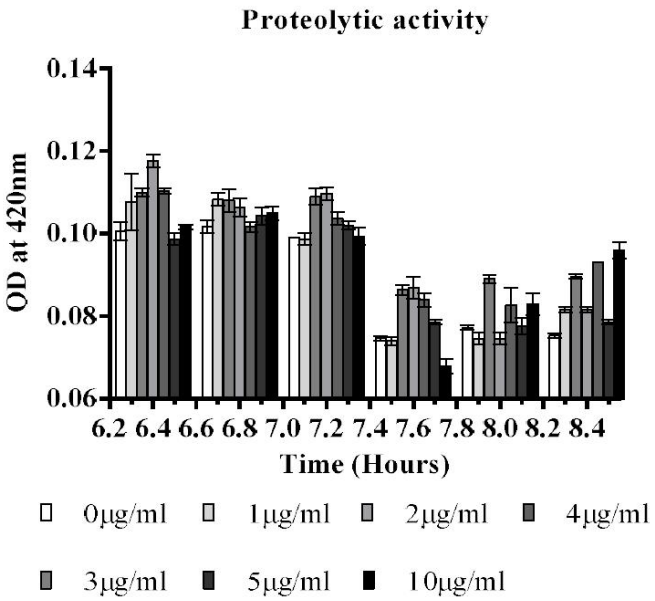

Figure 5 : Time and concentration dependent analysis of protease activity

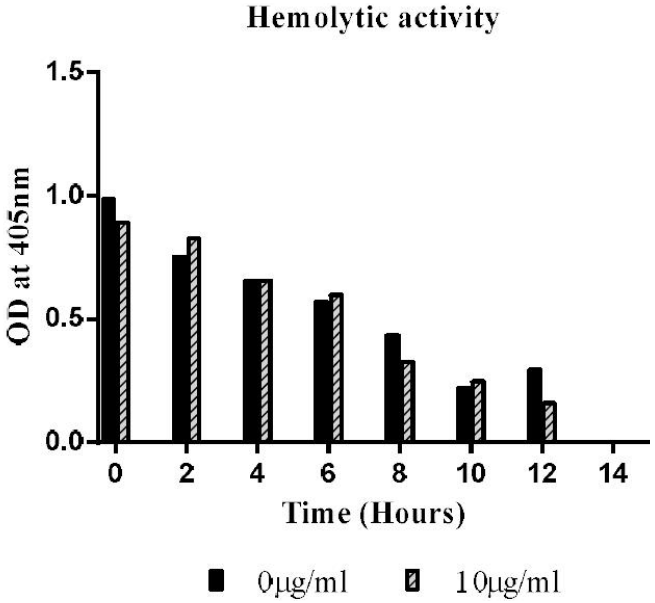

Figure 6 : Time and concentration dependent analysis of the haemolytic activity

main that represses hemolysin. Potential lead obtained through docking studies showed expected binding which can be inferred such a way that the potential compounds present in the extract can regulate the SmcR as expected. This was also verified through in vitro validation using the fungal extract which gave results similar to the docking studies. This states that the compound Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) VVSI3 can be used as potential drugs against *Vibrio vulnificus* infections.

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