

Isolation and characterization of marine bioluminescent bacteria for toxicity bioassays and biotechnological applications

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Abstract

Toxic heavy metals pollution posed severe health hazards to the environment and biodiversity. Therefore, development of rapid and non-invasive bioassays are in race to monitor toxic chemicals using novel approaches. This study isolated and characterized an intense blue luminescence producing marine bacteria, *Vibrio campbellii* STF1, for biosensing applications. Species-level identification of this strain was confirmed based on various phenotypic tests and multilocus sequence approach using 16s rRNA, *toxR*, and *luxA* gene sequence analysis. Fatty acid methyl ester analysis revealed the presence of three predominant fatty acids C_{15:0 anteiso} (21.73%), C_{17:0 anteiso} (11.27%), and C_{19:0 anteiso} (9.08%) in STF1. Luciferase enzyme from *V. campbellii* STF1 was extracted, partially purified, and molecular masses (alpha subunit 40 kDa and beta subunit 37 kDa) was determined by SDS-PAGE gel for *in vivo* assays. MALDI-TOF-MS analysis of *V. campbellii* cells' protein extracts showed distinct mass spectral peaks at m/z of 2615, 3948, and 4232 da. *V. campbellii* STF1 is resistant to heavy metal lead, while other metals such as cadmium, copper, and mercury inhibited its growth and luminescence. Crude ethyl acetate extraction of *V. campbellii* demonstrated antibacterial activity against *Shigella dysenteriae* type 5 with a maximum inhibition zone of 27.0±1.0 mm.

Keywords: *Vibrio campbellii*; multilocus sequence analysis; biosensor; heavy metal toxicity; antibacterial activity.

Introduction

Bioluminescent bacteria are one among the known mesmerizing luminous organisms on this tiny blue planet earth ¹. The distribution and diversity of these bacteria in various marine abiotic and biotic samples are widely studied from different environments ^{2,3}. Luminescence of these bacteria is regulated by structural and core *lux* genes ⁴ and by various physico-chemical factors ⁵. Quorum sensing—a chemical signalling phenomenon in which, luminous bacteria release a high concentration of quorum sensing molecules into the growth media, which triggers core genes *luxCDABE* to produce blue-green luminescence ⁶. The emission spectrum of all known bacterial bioluminescence falls within the range of 450-490 nm. Isolation of intense luminescence producing bacteria is current research interest in developing luminescence-based rapid bioassays to monitor various pollutants from various biological and environmental samples. On the other hand, the luciferase enzyme is also used as a natural *in vitro* biosensor to monitor several toxic effluents. Thus the whole bacteria and enzyme are being used in a myriad of ways, especially in biomedical and toxicological studies ^{7,8}.

Several recent studies emphasize the increased pollution levels in the atmosphere, hydrosphere, and lithosphere, indicating an alarm to detect and monitor these toxic pollutant levels. In order to monitor and detect pollutants from different environments, studies have been redirected to explore potential novel resources like luminous bacteria. A luminous *Vibrio campbellii* strain LZ5 isolated from the Pacific oyster, *Crassostrea giga*, was found to show the potential application in monitoring different heavy metals ⁹. For the first time, recent studies observed the bioactive nature of different species of luminous bacteria in the form of antimicrobial activity against various human pathogens ¹⁰. Identification of vibrios based on various phenotypic tests, fatty acid methyl esters analysis, and phylogenetic analyses using multilocus sequence analyzation (MLSA) approach was found to be reliable in current research ¹¹. Therefore, considering luminous bacteria's important applications, the phenotypic and genotypic characterisation was carried on the potential biosensing and bioactivity showing luminous marine bacteria *Vibrio campbellii* STF1.

Materials and methods

Study area and sample collection

During low tide time, a live specimen of unscheduled stonefish *Synanceia verrucosa* was caught carefully using a small hand net from Kodyaghat coast (Lat 11°31'47.16'' N; Long 92°43'25.97'' E). Specimen was immediately transferred to a sterile ziplock sample bag and transported to the laboratory within one hour for further microbiological analysis.

Isolation of luminous bacteria

All microbiological tests were performed according to standard aseptic techniques in microbiology¹². The collected specimen was washed thoroughly with autoclaved sterile seawater to remove epibionts and debris. Using a sterile cotton swab, the specimen's surface skin was swabbed and spread evenly onto SWC agar media Petri plate. Plate was incubated at 35°C for 24 hours and examined for luminous colonies under dark conditions by straining the eyes for 10 minutes. Using adjustable red light, intense luminous colonies were picked up with sterile toothpicks³, and purified by streaking on SWC agar plates until pure isolated colonies were obtained. Luminous strain with high intense luminescence was coded as STF1 and deposited in the host institution for further studies.

*Phenotypic characterization of luminous bacterium *V. campbellii* STF1*

Morphological study

Overnight grown pure culture of *V. campbellii* in the marine broth was used to observe morphology and motility under ZEISS Axiovert 40 CFL inverted microscope. A loop full of broth culture was placed on a clean glass slide and observed under a microscope at 40x magnification using a blue filter.

Scanning Electron Microscopic (SEM) analysis

V. campbellii was fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer–0.1 M sucrose (pH 7.4), rinsed thrice with 0.1 M phosphate buffer, and used for the fixative for 5 minutes after each rinse. Sample was postfixated for 1 hour in 1% osmium tetroxide in the same buffer, rinsed thrice with the same buffer for 5 minutes. The sample was dehydrated by adding ethanol solutions in a graded series of 50%, 70%, 80% for 10 minutes at each concentration, 95% was used twice for 10 minutes, and 100% was used thrice within 15 minutes. Sample was then dried overnight in a fume hood and mounted on aluminum stubs and sputter-coated with gold for observation in a VEGA3 TESCAN SEM.

Biochemical tests

Biochemical tests were performed according to standard techniques¹³ and using readymade discs (Himedia labs, Mumbai). All tests were performed according to both the protocols and with an optimum salt level (3% NaCl) used in all these tests. Results were compared with biochemical keys

¹⁴.

Fatty acid methyl esters profile analyzation

Extraction of fatty acid methyl esters (FAME) was followed according to the method described by Sasser ¹⁵. Gas Chromatography (GC) analysis of extracted fatty acids was carried out in Agilent technologies, model 6890N network with flame ionisation detector (FID) and high resolution gas chromatography column (Agilent Technologies) capillary with sizes 25m x 200µm x 0.33µm was used in this study. Sample was maintained for 21 minutes with injector temperature at 170°C and a detector temperature at 310°C. The carrier gas used was Hydrogen with a flow rate of 30 µl/min and 2µl of sample was used for the GC analysis with a split ratio of about 100:1. Quantification and identification of FAME peaks were determined with a reporting integrator model- Sherlock Microbial Identification System, version 6.0B.

MALDI-TOF mass spectrometry analysis

Protein analyzation of *V. campbellii* STF1 was performed using matrix assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) following the Tris-EDTA buffer approach method ¹⁶. Fresh culture of *V. campbellii* STF1 grown on Luria Broth agar (3% NaCl) for 24 hours at 37°C was used. Cells were harvested and transferred into eppendorf tube containing 2 ml of deionized water. This was vortexed and centrifuged at 4000 rpm for 5 minutes at room temperature (RT). The supernatant was decanted, and 1.5 ml of deionized water was added to the bacterial pellet. This was vortexed for 5 minutes and centrifuged at 19000 rpm for 30 minutes at RT. The supernatant was decanted, and 1.5 ml of TE buffer was added. Vortex was repeated for 20 minutes at RT and subsequently centrifuged at 19000 rpm for 30 minutes at RT. The pellet obtained was once again suspended with TE buffer and vigorously shaken for 10 minutes. This homogenous bacterial cell suspension of 0.5 µl was loaded in the MALDI target plate.

Growth curve determination

A growth curve study was performed to determine the luminescence activity with varied cell density. A 100 µl of overnight grown culture in seawater broth with an optical density (OD) value of 0.074 was inoculated into 50 ml seawater broth in a 100 ml conical flask and incubated at 35°C on a shaker with 150 rpm. For every one hour, OD values were measured at 600 nm using a spectrophotometer. Same broth without bacterial culture inoculation was used as a standard blank measurement.

Luminescence assay

For luminescence activity, 100 µl of fresh *V. campbellii* broth culture with an initial OD of 0.018 was inoculated in 50 ml luminescent broth and incubated at 35°C. Broth media without culture

inoculation was used as blank. Luminescence readings were recorded every one hour using the luminometer (Hidex).

Effect of pH on growth and luminescence

Tolerance to various pH levels was studied according to Benson ¹³. Different pH levels of 2, 5, 7, 9, and 11 were adjusted in 50 ml of seawater broth containing conical flasks using 1M NaOH and 1M HCl. Culture STF1 was inoculated in flasks, incubated in a shaker at 35°C for 24 hours, and arbitrary values were recorded.

Antibiotic sensitivity assay

Luminous bacteria are known to enter easily into aquaculture systems and cause luminous vibriosis disease, which results in dramatic losses in global aquaculture industries. Many studies have proven that these bacteria can resist antibiotics. To understand their resistance profile, different antibiotics were tested (Table S2). Antibiotic susceptibility assay was performed according to Benson ¹³. Various antibiotic discs were placed on freshly prepared Muller Hinton agar (3% NaCl) plates swabbed with overnight grown cultures in marine broth. Petri plates were incubated at 35° C for 24 hours, and results observed as inhibition zones were recorded.

Effect of heavy metals on growth and luminescence

In this study, the effect of heavy metals including Cadmium chloride, Copper sulphate, Lead nitrate, and Mercuric chloride on growth and luminescence and tolerance to these metals was studied at different concentrations of 0.1 mM, 1 mM and 10 mM. Heavy metal solutions were prepared according to Gellert ¹⁷, and heavy metal sensitivity assay was performed following Well diffusion method ¹⁸.

Extraction and purification of luciferase and visualization in SDS-PAGE

For more reliability of luciferase elucidation, two different methods have been employed for extractions from luminescent broth cultures ^{9,19}. Cell lysates obtained from both methods were centrifuged, and 5 ml of the supernatant was run through DEAE-52 column in respective elution buffer described in each method, and fractions were collected at a rate of 0.5 ml/min. The collected fractions from both methods were run in SDS-PAGE to visualize and determined luciferase enzyme molecular weights. The gel was run at 50 V and stained with Coomassie brilliant blue.

Extraction of secondary metabolites

Cultures of *V. campbellii* STF1 were grown in 30 ml of Marine Broth in 100 mL conical flasks, incubated in a shaker at 220rpm with continuous agitation for a period of 3 days at 28°C ²⁰. After the

incubation period, cultures were centrifuged at 10000 rpm for 15 minutes, and cells were removed. Supernatant of the cultures was extracted with an equal volume of ethyl acetate (EtOAc) and placed on a shaker at 220 rpm with continuous agitation for 12 hours to achieve a homogenous mixture. Subsequently, the organic phases were collected and concentrated with a Rota evaporator under reduced pressure at 37°C to remove solvents. The final crude extract obtained was redissolved in 1 ml EtOAc for antibacterial assay.

Antibacterial activity against human pathogenic bacteria

Antibacterial activity of STF1 was screened against 17 human pathogenic bacteria (Table 1). Stringent aseptic conditions were adopted for the preparation of inoculums of these pathogenic bacterial cultures. A loop full of each human pathogenic bacterial culture was inoculated into 5 ml glass tube containing nutrient broth medium and incubated at 35°C for overnight (12 to 18 hours) and used as inoculums suspension for antibacterial assay.

Antibacterial activity assay was performed according to Kirby-Bauer well diffusion method¹⁸. The inoculum suspensions of pathogenic bacterial cultures were swabbed onto entire surfaces of Mueller-Hinton agar plates. The lids of these swabbed Petri dishes were left ajar for 2 to 5 minutes until excess surface moisture was absorbed. Now wells were made on these plates with sterile cork borers, and 200 µl of the crude bacterial extract was impregnated into wells. The EtOAc solvent was used as a negative control. After complete diffusion of extract into media, plates were incubated at 35°C for 24 hours, and clear zones of inhibition formed around wells (including well size) were measured in diameters.

GC-MS analysis of crude extractions:

Crude extraction was analysed with GC-MS to determine compounds responsible for antimicrobial activity in this isolate. GC-MS analysis was performed with a Thermo GC – Trace Ultra Ver: 5.0, Thermo MS DSQ II, TR 5 – MS capillary standard non – polar column, injection volume 1 microliter, carrier gas Helium, with a flow rate of 1.0 ml/min.

Statistical analysis

Data results obtained from antibacterial assay and heavy metal sensitivity test were expressed as mean ± standard deviation using Microsoft Excel.

*Genotypic characterization of luminous bacterium *V. campbellii* STF1*

Genomic DNA isolation

Genomic DNA was isolated according to Cetyl Trimethyl Ammonium Bromide (CTAB) method²¹.

PCR amplification of luxA and toxR genes

For PCR amplification, two set of primers targeting the *luxA* 745 bp and *toxR* 477 bp were employed. PCR was carried out by using corresponding forward primer 5'-CTACTGGATCAAATGTCAAAGGACG-3' and reverse primer, 5'-TCAGAACCGTTTGCTTCAAACC-3' for *luxA* gene²²; and forward primer *toxRs* 5'-GANCARGGNTTYGARGTNGAYGAYTC-3' and reverse primer *toxRas* 5'-TTDKKTTGNCCNCYNGTVGCDATNAC-3' for *toxR* gene respectively²³. PCR of the genomic DNA of *V. campbellii* STF1 was conducted in a final volume of 25µl. The reaction mixture contained 10x PCR buffer (Himedia, Mumbai), 10 µM DNTP's, 1 U of Taq DNA polymerase, 10 µM of each forward and reverse oligonucleotide primers and approximately 20 ng of genomic DNA.

PCR amplification was performed using a GeneAmp PCR system 2720-ThermoCycler, (Applied Biosystem, Foster City, CA, USA). The amplification profile for *luxA* gene consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 58°C for 30 sec, 72°C for 30 min and a final extension step of 72°C for 7 min. For *toxR* gene, the thermal program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 7 min. Samples were held at 4°C until further analysis. Further PCR products were resolved in 1.2% agarose gel electrophoresis, gel stained with ethidium bromide and photographed with gel doc capture system.

Sequencing and phylogenetic analysis of luxA and toxR genes

Sequencing was performed with an automated Sequencer (Applied Biosystems, Foster City, USA) determined by Sanger dideoxynucleotide chain termination method. Prior to sequence, PCR products of *toxR* and *luxA* genes were purified with GeNoRime PCR Purification kit (Shrimpex, Chennai) and partially sequenced. The nucleotide sequences obtained were compared with sequences in public gene databases GenBank, EMBL, and DDBJ. Black Box Chimera Check (B2C2) software was used to check chimera formations²⁴. Alignment of sequences was carried out using MEGA 6 software²⁵, and uncertain alignments found at the ends of the sequences were omitted for further analyses. The same software was used to construct a phylogenetic tree. In order to construct and evaluate the reproducibility of the phylogenetic tree, the Neighbour-Joining method, Jukes-Cantor distance matrix, and 1000 bootstrap resamplings were accomplished.

Plasmid DNA Isolation

In order to confirm the presence of plasmid in *V. campbellii* STF1, plasmid DNA extraction was performed with a plasmid DNA extraction kit (Himedia, Mumbai). Plasmid DNAs were visualised in 1.2% agarose gel stained with ethidium bromide and visualised under UV gel doc system.

Results

Vibrio campbellii isolated from the surface of stonefish *S. verrucosa* has shown intense blue luminescence. Luminescence was more intense in broth culture than on the agar plates (Fig. 1).

Phenotypic characterization of V. campbellii STF1

Morphological observations revealed *V. campbellii* as short rods and motile (Fig. 1). Colonies are large in size with an entire margin, circular form, and convex elevation. This bacteria is Gram negative, green in colour on TCBS agar, and positive to oxidase, catalase, ONPG, O/129 vibriostatic agent (2,4-diamino-6,7-diisopropyl pteridine sulphate), nitrate reduction, citrate utilisation, methyl red test, and positive to various carbohydrates (Table S1). This strain is also positive for dye uptake. The maximum tolerable temperature was 40°C, and it even can tolerate 10% NaCl. Based on the biochemical identification keys, strain STF1 was identified as *V. campbellii*. This bacterium produces a non-diffusible brownish-orange pigment on marine agar (Fig. S1).

Major cellular fatty acids observed were saturated C_{15:0} anteiso, C_{17:0} anteiso, C_{19:0} anteiso, and C_{13:0} iso 3OH; unsaturated C_{18:1} ω9c, and summed C_{18:1} ω6c/C_{18:1} ω7c. Summed feature 2 comprises C_{14:0} 3OH and/or C_{16:1} iso I, summed feature 3 comprises C_{16:1} ω6c and/or C_{16:1} ω7c, summed feature 8 comprises C_{18:1} ω6c and/or C_{18:1} ω7c and summed feature 9 comprises C_{17:1} iso ω9c, and/or C_{16:0} 10-methyl. Various other fatty acids such as C_{11:0}, C_{12:0}, C_{11:0} 3OH, C_{14:1} iso, C_{14:0}, , C_{15:0} iso, C_{14:0} iso 3OH, C_{16:1} ω6c, C_{16:1} ω7c, C_{16:1} iso H, C_{16:0} iso, C_{16:0}, C_{15:0} iso 3OH, C_{17:1} iso ω5c, C_{17:0} iso, C_{17:1} ω5c, C_{17:0}, C_{17:0} iso 3OH, C_{18:0}, C_{18:0} 10-methyl, C_{19:0} iso, and C_{19:0} cyclo ω8c have also been observed in *V. campbellii* STF1. MALDI-TOF analysis showed distinct mass spectral peaks at mass-to-charge ratio (m/z) of 2615, 3948, and 4232 Da (Fig. S2). Further analysis is yet to be investigated to characterize the proteins responsible for these distinct peaks.

Log phase was observed during 1st hour of incubation and continued till 6th hour. The maximum intensity of luminescence produced by the luciferase enzyme was 8,320,627 photons per second observed at the eighth hour (Fig. 2a & 2b). Growth and luminescence of *V. campbellii* were less at pH < 7 and > 11, and optimum was found at pH 9 (data not shown). *In vitro*, antibiotic sensitivity test showed that this strain is sensitive to most tested antibiotics (Table S2), while resistant to amoxyclave, ampicillin, gentamicin, kanamycin, oxacilin, and penicillin. *V. campbellii* is

sensitive to mercury and cadmium at concentrations of 0.1 mM, 1 mM, and 10 mM with inhibition zones of 10.0 ± 0.0 mm, 18.0 ± 0.0 mm and 29.0 ± 0.0 mm, and 10.0 ± 0.0 mm, 13.0 ± 0.0 mm, 21.0 ± 0.0 mm respectively (Fig. 3; Table 2). It is resistant to lead metal. The molecular masses of partially purified luciferase enzyme alpha and beta subunits determined by SDS-PAGE gel were 40 kDa and 37 kDa, respectively (Fig. 4).

Crude ethyl acetate extractions stored in -20°C had exhibited good antibacterial activity against various human pathogenic bacteria (Table 1). Maximum zone of inhibition occurred against *Shigella dysenteriae* type 5 (27.0 ± 1.0 mm), and less activity was observed against *E. coli* (7.6 ± 0.5 mm). GC-MS chromatogram of crude extraction revealed two important compounds, tetradecanal (RT at 7.55), and 2-tert-Butyl-4-isopropyl-5-methylphenol (RT at 12.74) (Fig. S3).

Genotypic characterization of V. campbellii STF1

A total length of 745 bp corresponded to *luxA* gene was successfully amplified by PCR. The amplified *toxR* gene fragment size was larger (600 bp) than the expected size of 477 bp (Fig. 5). This sequence was aligned with database sequences and trimmed to 477 bp length, and analysed. Nucleotide sequences 16s rRNA, *luxA* and *toxR* genes were deposited in GenBank under the accession numbers of KF961190, KT372144, and KT354594, respectively. While the presence of plasmid indicated that *V. campbellii* antibiotic resistance is mediated by plasmid DNA.

Discussions

Vibrio campbellii strains are known to display morphological and genetic plasticity. Strain STF1 is found to show phenotypic and genotypic plasticity when compared with other strains of *V. campbellii* isolated previously from the Andaman waters¹⁰. Brown pigmentations observed in strain STF1 were not produced by other *V. campbellii* strains isolated in the previous study¹⁰. Brown pigment production of strain STF1 was similar to *V. campbellii* ATCC BAA-1116²⁶. A luminous bacterial strain, *V. campbellii* LZ5 isolated from the meat of *Crassostrea gigas* was reported to be resistant to two heavy metal ions, Cadmium and Mercury⁹. Conversely, in the present study, *V. campbellii* STF1 was sensitive to both the metals, indicating the intra species-specific resistance activity as well as the novelty of STF1. An earlier report by Ulitzur and Hastings suggested that tetradecanal is the natural aldehyde substrate involved in luminescence emission²⁷. In the present study also teradecanol was detected from strain STF1; thus this study is an evidence and supports the previous study observations²⁷.

Since phenol derivatives are well known as antimicrobial compounds, we speculate that the observed phenol derivative compound 2-tert-Butyl-4-isopropyl-5-methylphenol is likely being

responsible for the antibacterial activity of *V. campbellii*. A recent report revealed the potential antimicrobial activities of luminous bacteria against different human pathogens due to antibacterial compounds such as indole, phenol, 2,4-bis(1,1-dimethylethyl)-dibutyl phthalate, and 1,2-Benzenedicarboxylic acid, butyl octyl ester¹⁰. However, such compounds were not found in *V. campbellii* STF1. Significantly, strain-specific antimicrobial activity was displayed by *V. campbellii* strains such as BSE1, BSE5, BSECU1, BSECU3, and PEVII showed antibacterial activity against various pathogens¹⁰. Among these strains, BSECU1 displayed maximum inhibition zone of 24 mm against *Bacillus subtilis*¹⁰. Whereas the present study strain STF1 showed maximum inhibition zone against *Shigella dysenteriae* type 5 (27.0 ± 1.0 mm). But strains BSE1, BSE5, BSECU1, BSECU3, and PEVII did not show any antibacterial activity against *S. dysenteriae*¹⁰, indicating the distinct characteristics.

The distinct mass spectral peaks obtained for *V. campbellii* STF1 were assigned as specific biomarkers, which will serve as species-specific biomarkers for further identification. Multilocus sequence analysis using 16s rRNA⁵, *toxR* and *luxA* genes have identified strain STF1 as *V. campbellii*. This strain BLAST analysis of *toxR* gene sequence of *V. campbellii* STF1 revealed 99% sequence identity with *V. campbellii* VH1 (accession JX442514) isolated from *Litopenaeus vannamei* shrimp farm²⁸. *V. campbellii* VH1 and *V. campbellii* STF1 are both haemolytic and STF1 is different from VH1 as it is resistant to gentamicin, and kanamycin. While *luxA* gene sequence of *V. campbellii* STF1 showed 99% sequence identity with *V. campbellii* ATCC BAA-1116²⁶. In NJ tree also clustering was apparent with these two strains- VH1 and ATCCBAA-116 as similar to BLAST analysis (Fig. 5).

This study shows that luminous *V. campbellii* STF1 can be readily used as a natural biosensor *in vitro* to monitor different toxic metals and pollutants from food and agriculture industries. The various concentrations of pollutants can be prepared as similar to our study or earlier study⁹ and tested against luminous bacteria to generate luminescence excitation and inhibition profiles associated with each concentration of the interested pollutant. While the extracted luciferase enzyme from the strain STF1 can be employed in luminescence-based assays along with reduced flavin mononucleotide (FMNH₂) as luciferin to test pollutants from various environments. Either way, whole bacteria (strain STF1) and its luciferase enzyme would serve as a readily available *in vitro* biosensors to monitor a wide range of pollutants. Natural luminous strain over cloned luminous bacteria is advantageous because natural luminous bacteria produce intense luminescence than cloned bacteria. Developing cloned bacteria with *lux* genes is cost-effective, time-consuming, and uncertain with the luminescence intensity production. Thus, natural strains with intense

luminescence such as strain STF1 are more important in developing rapid toxicological assays. *V. campbellii* STF1 is also a potential bioactive compound producing marine luminous bacteria for biomedical applications.

Conclusion

The morphological and genetic variations observed within the *Vibrio campbellii* strains isolated from the Andaman islands suggest that these strains could be novel. Thus, whole genome analysis are required to understand their genome plasticity in the Andaman waters. The emission patterns of luminescence of *V. campbellii* STF1 under various toxic heavy metals promises its potential application in developing real-time biosensor in the laboratory. The identified phenol compounds may be extracted for drug application.

Conflict of Interest

The authors do not have any conflict of interest to declare.

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References

1. Wilson T, Hastings JW. *Bioluminescence: Living Lights, Lights for Living*. Harvard University Press; 2013.
2. Dunlap P V, Urbanczyk H. Luminous bacteria. In: Rosenberg E, DeLong EF, Thompson F, Lory S, Stackebrandt E, eds. *Prokaryotes*. 4th ed. Springer-Verlag; 2013:495-528.
3. Ramesh CH, Mohanraju R. Genetic diversity of bioluminescent bacteria in diverse marine niches. *Indian J Mar Sci*. 2017;46(10):2054-2062.
4. Dunlap P V. Biochemistry and genetics of bacterial bioluminescence. In: Thouand G, Marks R, eds. *Bioluminescence: Fundamentals and Applications in Biotechnology, Vol 1*. Springer-Verlag; 2014:37-64.
5. Ramesh CH, Mohanraju R, Murthy KN, Karthick P, Narayana S. Impact of light, temperature, salinity and glycerol on the intensity of luminescence and growth of marine bioluminescent bacteria *Vibrio campbellii* (strain STF1). *Curr Sci*. 2014;106(4):511-513.
6. Ramesh CH. Studies on marine bioluminescent bacteria from Andaman Islands. Published online 2016.
7. Shimomura O. *Bioluminescence: Chemical Principles and Methods*. World Scientific; 2006.
8. Ramesh CH. Terrestrial and marine bioluminescent organisms from the Indian subcontinent: a review. *Environ Monit Assess*. 2020;192:747.
9. Liu Z, Zhao J, Liang X, et al. Characterization of luminescent *Vibrio campbellii* LZ5 and its potential application in the detection of environmental heavy metals. *Biotechnol Appl Biochem*. 2012;59:405-410.
10. Ramesh CH, Mohanraju R. Antibacterial activity of marine bioluminescent bacteria. *Indian J Mar Sci*. 2017;46(10):2063-2074.
11. Gomez-Gil B, Thompson CC, Matsumura Y, et al. Family Vibrionaceae. In: Rosenberg E, DeLong EF, Thonpson FL, Lory S, Stackebrandt E, eds. *The Prokaryotes*,. 4th ed. Springer-Verlag; 2014:659-747.
12. WHO. *Laboratory Biosafety Manual*. 3rd ed. World Health Organization; 2004.
13. Benson. *Microbiological Applications Lab Manual*. 8th ed. The McGraw–Hill Companies; 2001.
14. Noguerola I, Blanch AR. Identification of *Vibrio* spp. with a set of dichotomous keys. *J Appl Microbiol*. 2008;105:175-185.
15. Sasser M. *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*. MIDI Technical Note 101; 1990.
16. Ahmad F, Wu H. High-resolution MALDI-TOF mass spectrometry of bacterial proteins using a Tris-EDTA buffer approach. *Microchim Acta*. 2012;176:311-316.

17. Gellert G. Sensitivity and significance of luminescent bacteria in chronic toxicity testing based on growth and bioluminescence. *Ecotoxicol Environ Saf.* 2000;45:87-91.
18. Bauer AW, Kirby WMM, Sherris JS, Turk M. Antibiotic susceptibility by standardized single disc method. *Am J Clin Pathol.* 1966;45:493-496.
19. Hastings JW, Baldwin TO, Nicoli MZ. Bacterial luciferase: assay, purification and properties. *Methods Enzymol.* 1978;57:135-152.
20. Wietz M, Mansson M, Gotfredsen CH, Larsen TO. Antibacterial compounds from marine Vibrionaceae isolated on a global expedition. *Mar Drugs.* 2010;8:2946-2960.
21. Nishiguchi MK, Doukakis P, Egan M, et al. DNA Isolation Procedure. In: R. DeSalle et al., ed. *Methods and Tools in Biosciences and Medicine Techniques in Molecular Systematics and Evolution.* Birkhäuser Verlag; 2002:249-287.
22. Wimpee C, Nadeau TL, Neelson K. Development of species-specific hybridization probes for marine luminous bacteria by using in vitro DNA amplification. *Appl Environ Microbiol.* 1991;57:1319-1324.
23. Pascual J, Macian MC, Arahal DR, Garay E, Pujalte MJ. Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, recA, pyrH, rpoD, gyrB, rctB and toxR genes. *Int J Syst Evol Microbiol.* 2010;60:154-165.
24. Gontcharova V, Youn E, Wolcott RD, Hollister EB, Gentry TJ, Dowd SE. Black box chimera check (B2C2): a windows-based software for batch depletion of chimeras from bacterial 16S rRNA gene datasets. *Open Microbiol J.* 2010;4:47-52.
25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol.* 2013;30(12):2725-2729. doi:10.1093/molbev/mst197
26. Wang Z, Lin B, Mostaghim A, et al. *Vibrio campbellii* hmgA-mediated pyomelanization impairs quorum sensing, virulence, and cellular fitness. *Front Microbiol.* 2013;4:1-11.
27. Ulitzur S, Hastings JW. Evidence for tetradecanal as the natural aldehyde in bacterial bioluminescence. *PNAS.* 1979;76:265-267.
28. Wang L, Chen Y, Huang H, Huang Z, Chen H, Shao Z. Isolation and identification of *Vibrio campbellii* as a bacterial pathogen for luminous vibriosis of *Litopenaeus vannamei*. *Aquac Res.* 2013;46:395-404.

Figures & legends:

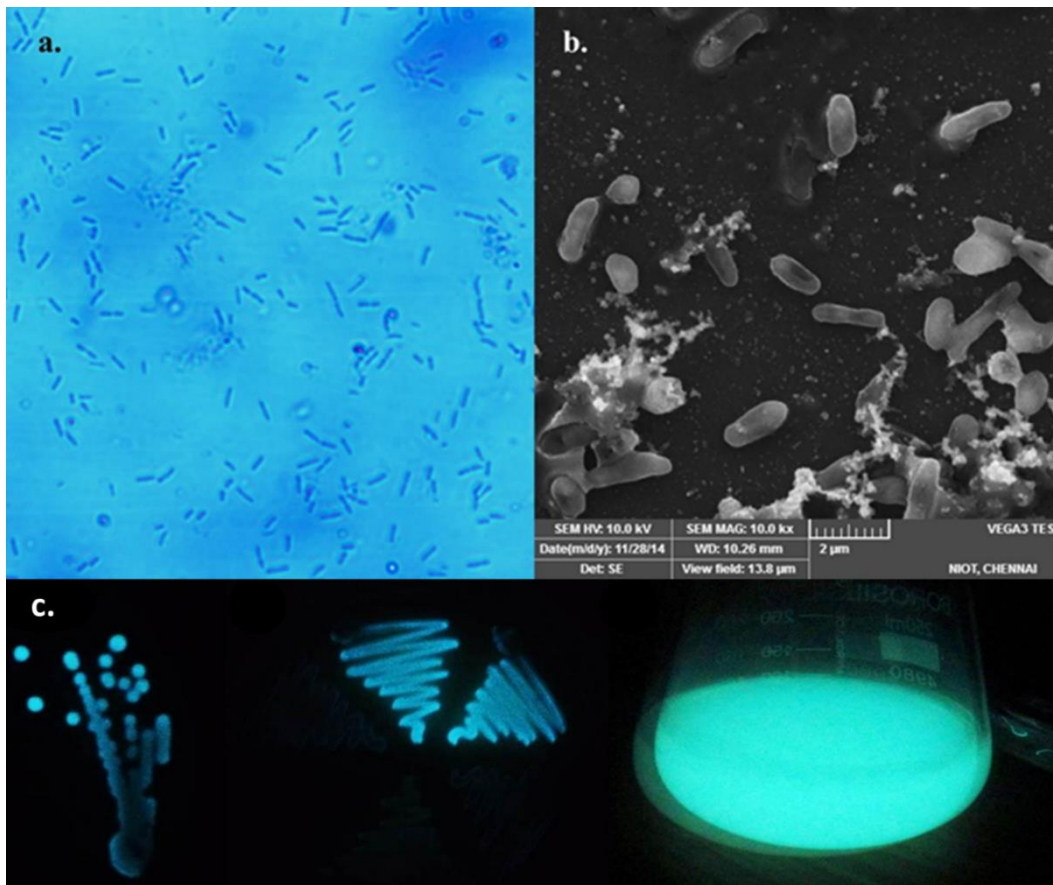


Fig. 1 Rod shape morphology of *V. campbellii* observed under inverted microscope (a) and SEM (b); Luminescence on SWC agar plate (left), marine agar (middle) and luminescent broth (right) (c).

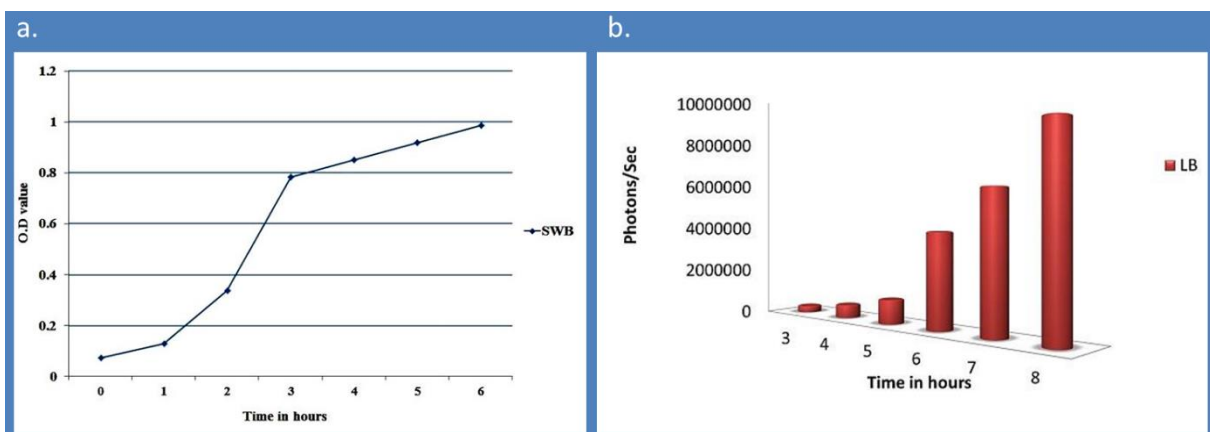


Fig. 2 Growth curve (a) and luminescence count (b) of *V. campbellii*.

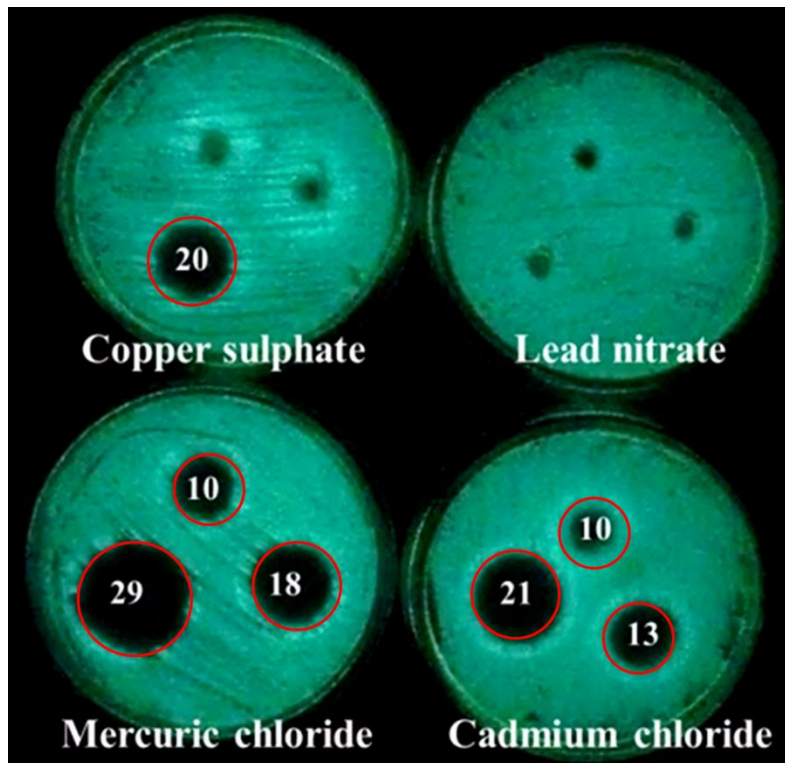


Fig. 3 Sensitivity of STF1 to different heavy metals. Red line rings indicate inhibition zone and numbers are inhibition zones in diameter.

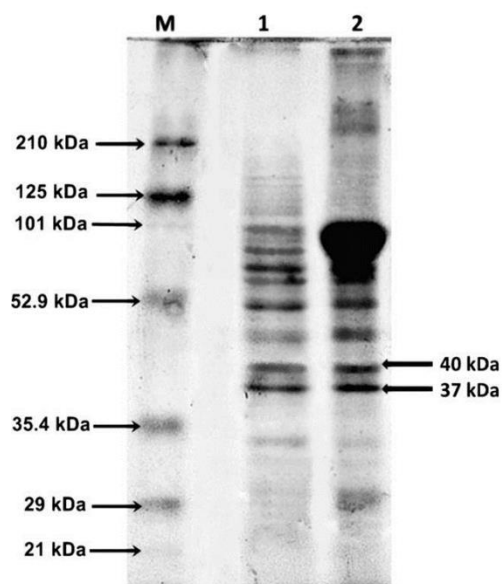


Fig. 4 Visualization of *V. campbellii* luciferase enzyme alpha (40 kDa) and beta (37 kDa) subunits in SDS-PAGE gel. Lanes: M, marker; 1, method of Hastings et al. (1978); 2, method of Liu et al. (2012).

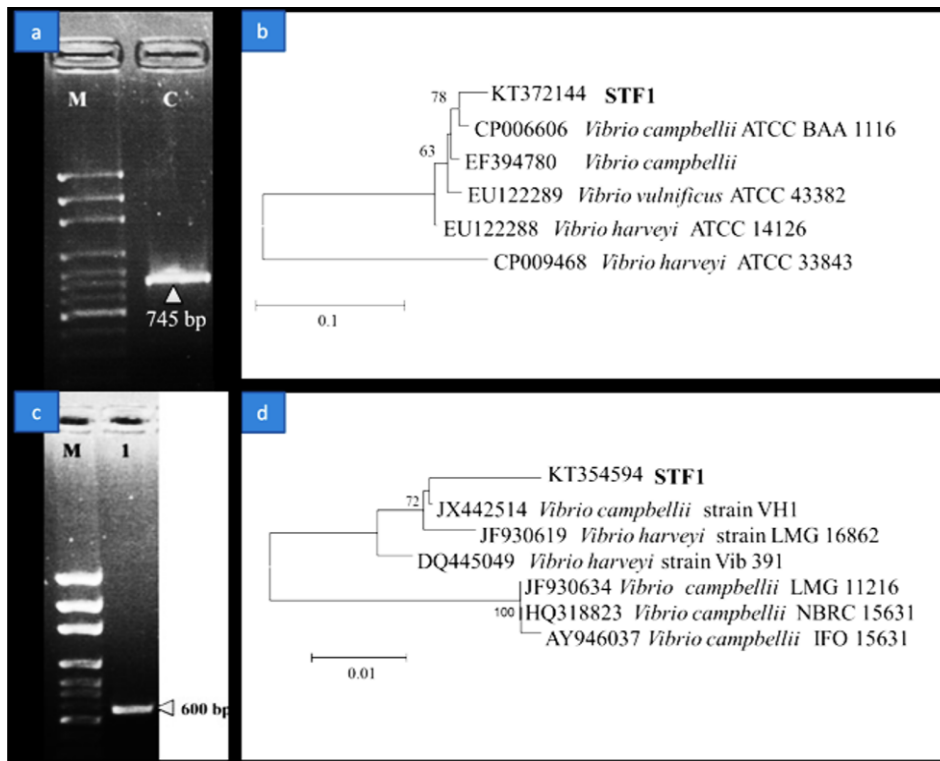


Fig. 5 *LuxA* gene PCR products visualised under gel doc system. Lanes: M, 100 bp marker; C, amplified *luxA* gene product of 745 bp; **(a)** NJ tree of *luxA* gene of STF1, **(b)** *ToxR* gene PCR products. **(c)** Lanes: M, 100 bp marker; 1, amplified *toxR* gene; **(d)** NJ tree of *toxR* gene of STF1.

Table 1. Antimicrobial activity of *V. campbellii* STF1 against different human pathogenic bacteria.

S. No	Human pathogenic bacteria	Growth Inhibition zones (Mean \pm Standard Deviation)
1.	Enterotoxigenic <i>Escherichia coli</i> serotype O115	7.6 \pm 0.5 mm
2.	Enteropathogenic <i>E.coli</i> serotype O114	7.6 \pm 0.5 mm
3.	Shiga toxin producing <i>E.coli</i> serotype O157:H7	8.6 \pm 0.5 mm
4.	<i>Klebsiella pneumoniae</i>	15.5 \pm 0.5 mm
5.	<i>Pseudomonas aeruginosa</i>	-
6.	<i>Salmonella typhi</i>	-
7.	<i>Salmonella typhi</i> C6953	-
8.	<i>Salmonella typhi</i> B12101	8.6 \pm 0.5 mm
9.	<i>Staphylococcus aureus</i>	-
10.	<i>Staphylococcus aureus</i>	11.0 \pm 0.5 mm
11.	<i>Shigella flexneri</i> type 2a	9.0 \pm 0.5 mm
12.	<i>Shigella dysenteriae</i> type 5	27.0 \pm 1.0 mm
13.	<i>Shigella boydii</i> type 1	10.5 \pm 0.5 mm
14.	<i>Shigella sonnei</i>	8.6 \pm 0.5 mm
15.	<i>Aeromonas hydrophilla</i>	11.3 \pm 0.3 mm
16.	<i>Vibrio cholera</i> O139	12.3 \pm 0.5 mm

Table 2. Sensitivity of STF1 to different heavy metals (inhibition zones in diameter).

S. No	Heavy metal	Growth inhibition zones in different concentrations (Mean \pm Standard Deviation)		
		In 0.1 mM	In 1 mM	In 10 mM
1.	Cadmium chloride	10.0 \pm 0.2 mm	13.0 \pm 0.1 mm	21.0 \pm 0.3 mm
2.	Copper sulphate	-	-	20.0 \pm 0.1 mm
3.	Lead nitrate	-	-	-
4.	Mercuric chloride	10.0 \pm 0.1 mm	18.0 \pm 0.2 mm	29.0 \pm 0.4 mm