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Chemical and structural characterization of hydroxamate siderophore produced by marine *Vibrio harveyi*

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Abstract In the present study, 22 different bacteria were isolated from open ocean water from the Gulf of Mannar, India. Of the 22 isolates, 4 were identified as *Vibrio* spp. (VM1, VM2, VM3 and VM4) and found to produce siderophores (iron-binding chelators) under iron-limited conditions. Different media were found to have an influence on siderophore production. Maximum siderophore production was observed with VM1 isolate in MM9 salts medium at 48 h of incubation. The isolate was confirmed as *Vibrio harveyi* based on 16S rRNA gene sequencing and phylogenetic analysis. Fourier-transform infrared (FTIR) and ^{1}H nuclear magnetic resonance (NMR) spectra revealed the hydroxamate nature of the siderophore produced. Further characterization of the siderophore revealed it to be of dihydroxamate nature, forming hexadentate ligands with Fe(III) ions. A narrow shift in ultraviolet (UV)–Vis spectrum was observed on photolysis due to ligand oxidation. Growth-promotion bioassay with *Aeromonas hydrophila*, *Staphylococcus aureus* and *E. coli* confirmed the iron-scavenging property of the siderophore produced by *Vibrio harveyi*.

Keywords Iron chelation · Siderophore · Ligand denticity · Proton NMR · *Vibrio harveyi*

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Introduction

Iron is the fourth most common element in the Earth's crust [29]. Acquisition of iron from the environment is vital to all forms of life, as iron plays a key role in oxidation–reduction reactions, adenosine triphosphate (ATP) synthesis, detoxification of oxygen radicals, electron transport pathways and a variety of biochemical reactions [11]. Although iron is one of the most abundant crustal elements, it is scarcely available due to rapid oxidation of ferrous [Fe(II)] to ferric [Fe(III)] state. Ferric iron is highly insoluble in aerobic and aqueous conditions, making its acquisition by microorganisms (and indeed higher organisms) a considerable challenge [8].

The difficulty of iron acquisition is further compounded for marine microorganisms due to the low iron concentration in seawater. Dissolved iron in open ocean water is at sub-nanomolar (0.02–1 nm) concentration, much lower than the concentration (0.4–1 μm) required by microorganisms for growth [23]. To circumvent this extreme iron shortage, many marine bacteria have developed siderophore-mediated iron transport systems. Siderophores are low-molecular-weight (<1,000 D) multidentate ligands that scavenge iron [Fe(III)] from the environment and transport it into cells via iron-regulated outer membrane proteins (IROMP). Based on the chemical nature of their coordination sites, microbial siderophores are classified as hydroxamates, catecholates, carboxylates and mixed type (β-hydroxyl aspartate/catecholate) [1].

Iron chelators in seawater are predominantly of bacterial origin [37]. However, in contrast to siderophores produced by terrestrial microorganisms, knowledge about marine bacterial siderophores is relatively recent, and only a few reports are available [5]. Iron sequestration systems have been identified as an important virulence mechanism in several

pathogenic bacteria. Members of the *Vibrionaceae* family, especially the genus *Vibrio*, have a specific emphasis among these pathogenic organisms. The genus *Vibrio* is Gram negative, halophilic and causes gastroenteritis and wound infections [44]. *Vibrio* sp. is the aetiological agent of the lethal septicaemic disease known as vibriosis, which affects humans and a large number of fish species worldwide [28].

One of the virulence factors implicated in vibriosis is the ability to utilize iron from the immediate environment by means of an iron-sequestering system [18]. Little is known regarding the iron-acquisition system of marine *Vibrio* species, although previous studies have provided evidence that siderophore-mediated iron uptake exists [7]. The production or utilization of siderophores available in the environment is crucial for bacteria in overcoming iron-limited conditions [40]. The surface waters of the world's oceans are exposed to significant levels of radiation, which contributes to photolysis of the ligand, thereby serving to increase the net bioavailability of iron. Despite the obvious importance of siderophores, there are many unanswered questions regarding their production and nature [43]. Understanding the influence of nutrients on siderophore production by bacteria will have important implications. Therefore, the present work was designed to isolate siderophore-producing *Vibrio* species from a marine environment in order to investigate the influence of different media on siderophore production and determine the characteristics of the siderophore produced using spectral analyses.

Materials and methods

Sample collection and isolation of bacteria

Water samples were collected from open ocean coastal regions near Rameswaram, Gulf of Mannar under aseptic conditions. Samples were processed for bacteriological analysis within 4 h of collection. Samples were serially diluted with sterile, aged seawater, and 1 ml of the diluted samples was transferred to Zobell marine agar for bacterial isolation.

Identification of bacterial strains

A loopful of isolated bacteria was streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar (Hi Media) and incubated at 27°C for 48 h. Coloured colonies were picked and subjected to biochemical tests for identification of *Vibrio* species [4]. In addition to biochemical analyses a particular isolate (VM1) was subjected to 16S rRNA partial gene sequencing and phylogenetic analysis.

Isolate VM1 was grown in Zobell marine broth. Total DNA was extracted as per the procedure of Babu et al. [2]. Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-ACGGCTACCTTGTTACGACTT-3′ [39]. The 16S rRNA gene sequence of the isolate was compared with related gene sequences available at the National Center for Biotechnology Information (NCBI) database for sequence homology using the BLAST online service (www.ncbi.nlm.nih.gov/blast), and phylogenetic analysis was performed using CLUSTALW version 1.8 [36]. Selected sequences were aligned in BioEdit [12]. Phylogeny was examined by neighbour-joining dendrogram using the Molecular Evolutionary Genetics Analysis software package (MEGA version 4.0). The topology of the phylogentic tree was evaluated by the bootstrap resampling method [16].

Growth conditions and siderophore production

Isolates were grown in iron-depleted chemically defined minimal medium (MM9) for 24 h at 28°C with shaking (120 rpm). The medium was composed of 0.3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1.0 g l^{-1} NH_4Cl, 6.0 g l^{-1} NaOH and 30.24 g l^{-1} piperazine-*N*,*N*'-bis(2-ethanesulphonic acid) (PIPES). This solution was autoclaved and supplemented with 30 ml 10% (m/v) deferrated casamino acids (contaminated iron was removed with 3% 8-hydroxyquinoline in chloroform), 2.0 g l^{-1} glucose, 1 ml 1 M $MgCl_2$ and 1 ml 100 mM $CaCl_2$. These solutions were prepared and sterilized separately. After incubation, cultures were centrifuged at 10,000 rpm for 15 min and the cell-free supernatant was examined for presence of siderophore by $FeCl_3$ test, chrome azurol sulphonate (CAS) assay and CAS agar plate test [32]. The nature of the siderophore produced by the isolates was ascertained by examining absorption maxima (λ_{max}) of ferric–siderophore complex using a UV–Vis spectrophotometer. Furthermore, the supernatant was subjected to Csaky, Arnow, and Vogel's assay [27] to confirm the nature of siderophore produced.

Siderophore quantification in different media

Three different media were used for siderophore production, namely thiosulphate citrate bile salt sucrose (TCBS) broth [35], Zobell marine broth (ZMB) [35] and minimal medium [27]. Deionized water was used for media preparation. Each medium was separately inoculated with the selected isolates (24 h culture) at a rate of 1% (v/v) and incubated with shaking. Three replicates were maintained for each medium. Following incubation, growth was measured and siderophore units were quantified [27]. Data were statistically analyzed using analysis of variance (ANOVA).

Purification and recovery of siderophore

The supernatant of *Vibrio harveyi* (VM1) was acidified to pH 3.0 using HCl and passed through a column packed with Amberlite XAD-400. The siderophore was eluted with methanol after washing the column with four bed volumes of deionized water. The eluted fraction was tested for siderophore by thin-layer chromatography using butanol:acetic acid:water (12:3:5, v/v) solvent system and CAS assay. The fraction was concentrated under vacuum using a rotary evaporator (Buchi, R-124) and freeze-dried using lyophilizer (Bio-systems). The dried sample was then stored at −20°C [30].

FTIR analysis of purified siderophore

A Fourier-transform IR spectrum was recorded for siderophore produced by VM1 isolate after lyophilization. Lyophilized siderophore sample was pelleted with potassium bromide (KBr) and subjected to FTIR spectroscopy (Jasco 460 plus) for determination of functional groups. Spectra were recorded in the range from 4,000 to 400 cm^{-1} [26].

Proton NMR analysis of purified siderophore

The ^{1}H NMR spectra was collected on a BRUKER 500-MHz instrument equipped with triplet resonance probe and triple axis gradients. XAD-purified siderophore sample was dissolved in 0.75 ml deuterated dimethyl sulphoxide (DMSO) and injected through a 5-mm one-dimensional NMR tube to record the nuclear magnetic resonance, with solvent signal as internal reference [26].

Determination of hydroxamate nature

Mono-, di- or trihydroxamate nature of the hydroxamate siderophores was tested by spectrophotometric method and paper electrophoretic method [15]. Shift in λ_{max} as a function of pH value was used to distinguish the ferric complexes of hydroxamate siderophores into mono-, di- or trihydroxamates. The paper electrophoretic method was performed to further distinguish the ferric complexes. The electrophoresis was run at 30 V cm^{-1} for 1–2 h with 4% formic acid (pH 2). The paper was dried to remove traces of formic acid and sprayed on both sides with 2% $FeCl_3$ solution.

Ligand denticity of hydroxamate siderophore

Hydroxamate siderophores are further distinguished into bi-, tetra- and hexadentate on the basis of the number of bonds that the ligand forms with the metal ion. Ligand denticity of the hydroxamate siderophores was determined by their change in colour at different pH values (4–9). Ferric hexadentate type maintains a stable (red) colour over a wide range of pH. Bidentate and tetradentate hydroxamate siderophores exhibit a colour shift with varying pH [22].

Photolytic properties

Hydroxamate siderophore produced by *Vibrio harveyi* (VM1) was dissolved in 0.2-μm-filtered, ultraviolet (UV)-irradiated seawater (pH 8) to concentration of 10 μM (trace metals and trace-metal-binding organic compounds from the seawater were removed as described by Macrellis et al. [19]). The solution was placed in an acid-washed quartz flask and exposed to natural sunlight for 6 h between 10:00 and 16:00 h. Identical solutions shielded from sunlight served as dark control.

Bioassay

Growth-promotion assay with *Aeromonas hydrophila*, *Staphylococcus aureus* and *E. coli* was performed according to the modified method suggested by Winkelmann et al. [42]. Fifty microlitres from a 24-h culture was inoculated into freshly prepared MM9 soft agar. Iron chelator 2,2-dipyridyl (150 μM) was added to the soft agar to reduce the available iron in the medium. Purified siderophore fraction (5 μl) was pipetted directly onto the agar surface. Growth zones were read after 2 days of incubation at 37°C. This test is a fast and reliable bioassay for determining the siderophore activity of the compound concerned.

Results and discussion

Identification of bacterial isolates

Twenty-two phenotypically different bacterial strains were isolated during the study period. Of the 22 isolates, 4 were screened to be *Vibrio* species based on their growth on TCBS agar and by their sensitivity to vibriostatic agent. The four isolates were Gram-negative, motile rods, oxidase and catalase positive. The isolates were confirmed as *Vibrio* species based on their salt tolerance, *O*-nitrophenyl-*β*-D-galactopyranoside (ONPG) test, arginine dihydrolase test, ornithine decarboxylase test and other biochemical tests (results not shown). The isolates were designated as strains VM1, VM2, VM3 and VM4. *Vibrio* species in the open ocean were chosen in this study for three reasons: (a) the open ocean environment is relatively devoid of iron; (b) *Vibrio* species are some of the most common organisms in

surface waters throughout the world; and (c) they have been recognized as significant pathogens in marine environments [14].

Siderophore assays

Under iron-limited conditions, all four *Vibrio* isolates produced siderophores, as confirmed by positive $FeCl_3$ test, CAS and spectrophotometric assay. All isolates produced orange halos around colonies in CAS agar plate, further confirming siderophore production. The result of this study suggests that the *Vibrio* isolates rely on siderophore-based iron-sequestration mechanism. Several marine *Vibrio* species have been reported to produce siderophores for iron uptake [42].

Characterization of siderophores

Siderophore produced by isolates VM3 and VM4 was confirmed as catecholate by Arnow's test and spectrophotometric assay. Isolates VM1 and VM2 were found to produce hydroxamate- and carboxylate-type siderophores, respectively, as confirmed by spectrophotometric assay, tetrazolium salt test and Vogel's chemical test. Spectrophotometric analysis of the VM1 culture supernatant showed a peak at 409 nm, confirming its hydroxamate nature. The absorption maximum at 218 nm indicated the presence of a carboxylate-type siderophore. Cabaj and Kosakowska [6] indicated that catecholate siderophores predominate in the genus *Vibrio*. Production of hydroxamate siderophores by marine bacteria confirmed our previous report [25] and is in accordance with the report of Martinez et al. [21]. This study clearly indicates that it is not possible to characterize the siderophore based on the microbial genera.

Quantification of siderophores

Siderophore production at different time intervals is depicted in Fig. 1. The values are averages of three replicates. Of the three media tested, maximum siderophore

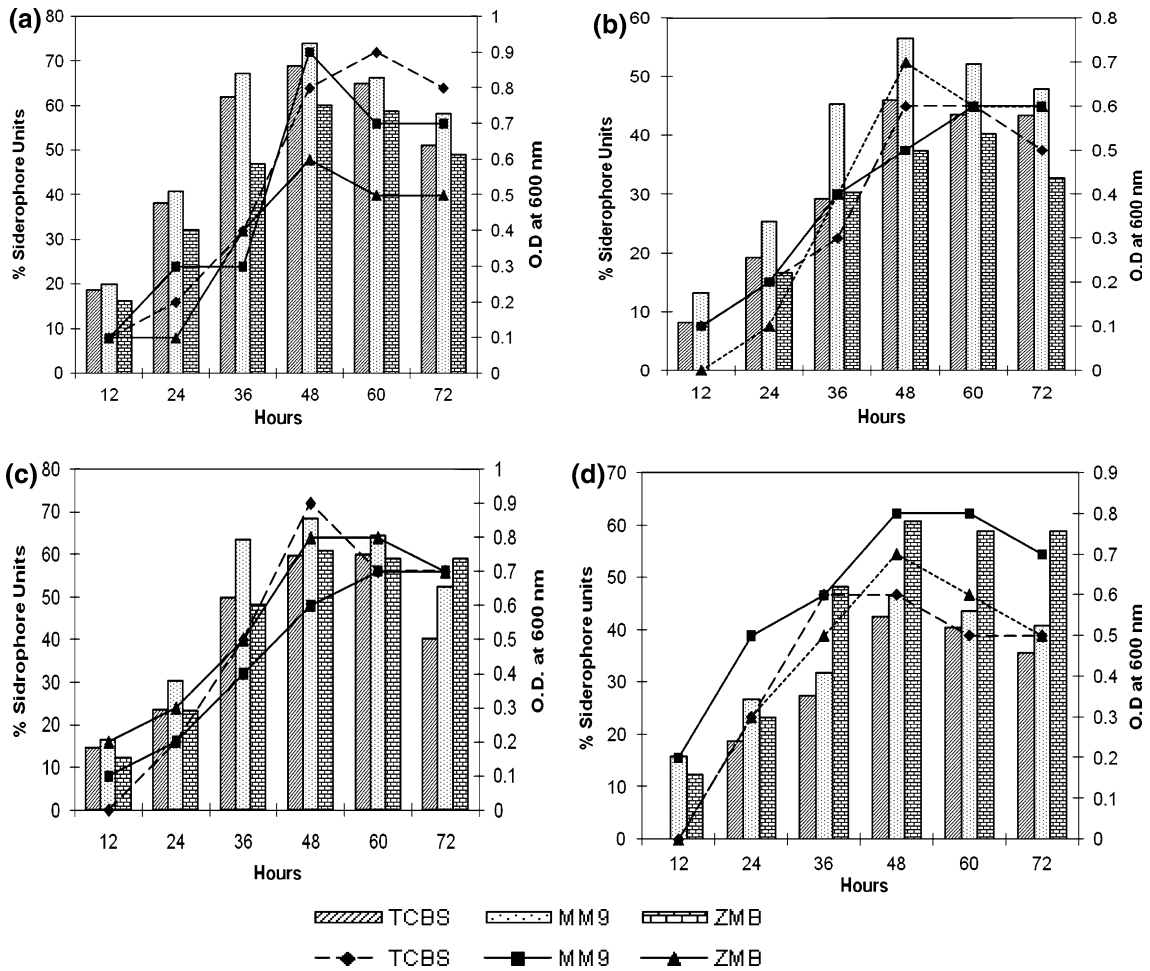

Fig. 1 Growth curve and siderophore production of *Vibrio* isolates VM1 (**a**), VM2 (**b**), VM3 (**c**) and VM4 (**d**) in different media: thiosulphate citrate bile salts sucrose (*TCBS*), minimal medium (*MM9*) and Zobell marine broth (*ZMB*)

production by all isolates was observed with MM9 salts medium compared with TCBS broth and ZM broth. Earlier studies revealed that the amount of siderophore production is substrate dependent [10]. In the present study, the maximum siderophore production in MM9 salts medium may be due to the presence of amino acids, $MgCl_2$, phosphates and low iron concentration, suggesting their importance in siderophore synthesis. Gram [10] reported that minimal medium bears little resemblance to the marine environment. Siderophore production by the different isolates on different media increased with incubation time up to 48 h and declined thereafter. The amount of siderophores produced by the bacterial isolates varied and was found to be positively related to their growth (Fig. 1). Difference in the quantity of siderophore production is a logical observation, and several reports have indicated variations in siderophore production with time, space and environment [31]. Yoshida et al. [45] reported the influence of iron on siderophore production. In the present study, statistical analysis of the data using three-way ANOVA revealed a significant difference ($P < 0.05$) in siderophore production between isolates (cumulative difference (CD) = 0.80), media (CD = 0.69) and incubation time (CD = 0.97). Furthermore, the difference was also significant (CD = 3.36) for the interaction effect between isolates, media and incubation time.

Low availability of iron is renowned in seawater due to its rapid oxidation to insoluble ferric state, thus becoming biologically unavailable. Therefore, it is not surprising that siderophore production is high in marine isolates. In the present study the highest product yield was obtained with VM1 isolate in all three tested media. Therefore, we decided to carry out further study on this particular isolate. Based on the morphological and biochemical characteristics and by their growth on *V. harveyi* agar (VHA), the isolate was identified as *Vibrio harveyi* [13]. This was further confirmed by 16S rRNA gene sequencing (available under NCBI accession no. GQ249053).

Phylogenetic analysis

The phylogenetic tree generated by the neighbour-joining method is shown in Fig. 2. It is clear from the phylogenetic analysis that strain VM1 is a member of the genus *Vibrio*. Bootstrap values also support inclusion of strain VM1 in the genus *Vibrio*. Moreover the tree topology suggests that the strain is most closely related to *Vibrio harveyi*. The dendrogram shows reliability of branching order, based on bootstrap analysis. Bootstrap values of 50% or greater are considered reliable [38].

Siderophore purification

Acidified supernatant passed through Amberlite XAD-400 was eluted with methanol. The eluted fraction was vacuum-dried and dissolved in autoclaved deionized water (1 mg/ml). Orange colour formation with CAS solution indicated presence of siderophore in the eluted solution. Hydroxamate nature was ascertained by positive Csaky test. The purity of the siderophore was confirmed by visible wine-coloured spot formation on silica gel plates (data not shown) on thin-layer chromatography.

Chemical characterization

Functional group determination by FTIR

IR spectra of the hydroxamate siderophore showed a peak at 3,415.31 cm^{-1}, indicating the presence of a primary alcoholic group. A strong peak at 1,042.34 cm^{-1} indicates the presence of a primary aliphatic alcoholic group. The result of this study is in accordance with Patel et al. [26], who observed a similar hydroxyl peak at 3,357.21 cm^{-1}. The peaks at 2,360.44, 1,620.88 and 1,407.78 cm^{-1} may be due to C=N stretch, C–OH stretch and C=O stretch, respectively (Fig. 3). The lowering of the C=O stretch peak may be due to conjugation as well as intramolecular hydrogen bonding.

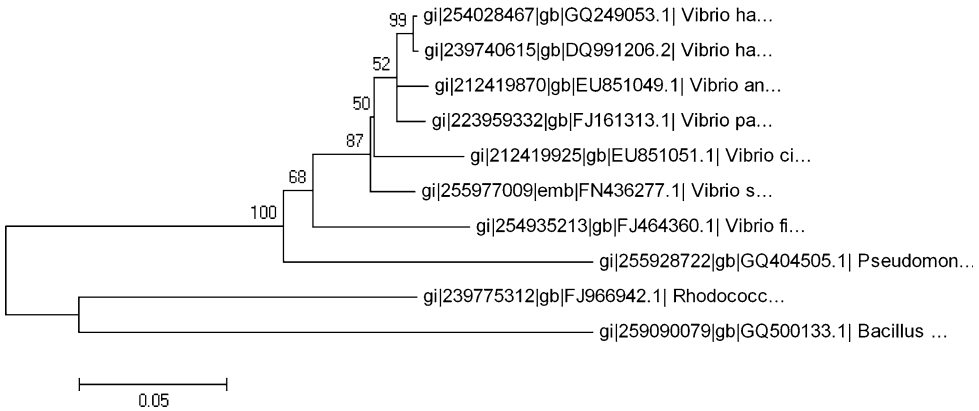

Fig. 2 Phylogenetic analysis (*neighbour-joining dendrogram*) of 16S rRNA gene sequences of the isolated *Vibrio harveyi* (VM1; GI 254028467) and related published sequences (given with GenBank accession numbers). Bootstrap values are shown near the clades

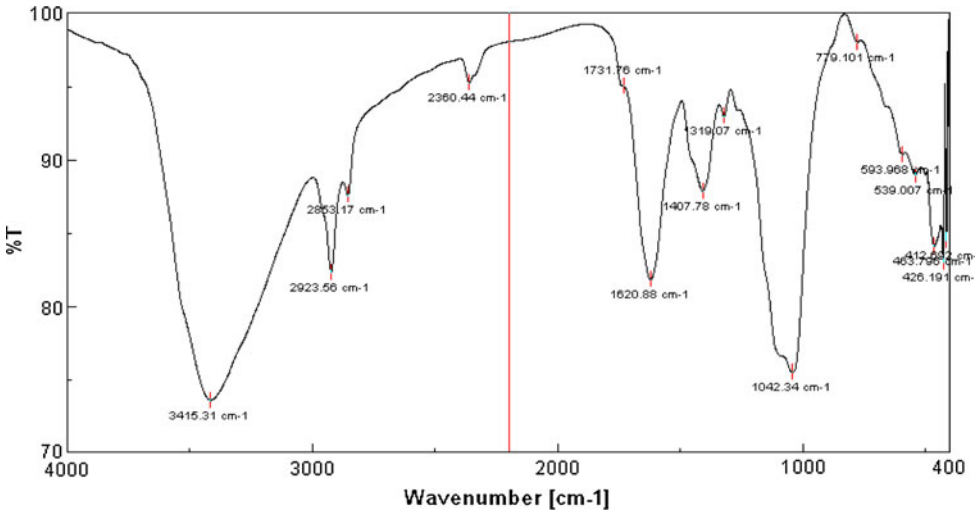

Fig. 3 Infrared spectrum of purified siderophore produced by *V. harveyi* (VM1)

^{1}H NMR spectroscopy

A proton NMR spectrum of hydroxamate siderophore produced by *Vibrio harveyi* taken in deuterated dimethyl sulphoxide (DMSO) exhibited three triplet peaks at 0.84, 0.91 and 2.18 ppm due to **CH_2**$-CH_2$, CH_2-**CH_2** and CH_2=**CH**, respectively. A doublet peak at 1.27 ppm indicates **CH_2**=CH, and three singlet peaks at 1.35, 1.25 and 3.37 ppm correspond to CH_2OH, =CH and aliphatic OH. The resonance at 2.50 ppm is considered a solvent peak (Fig. 4). The ^{1}H NMR spectrum of the present study establishes the presence of a dihydroxamate-type siderophore. These spectral results coincide with those of dihydroxamate siderophore (bisucaberin and aerobactin) produced by marine *Vibrio* species [24, 42].

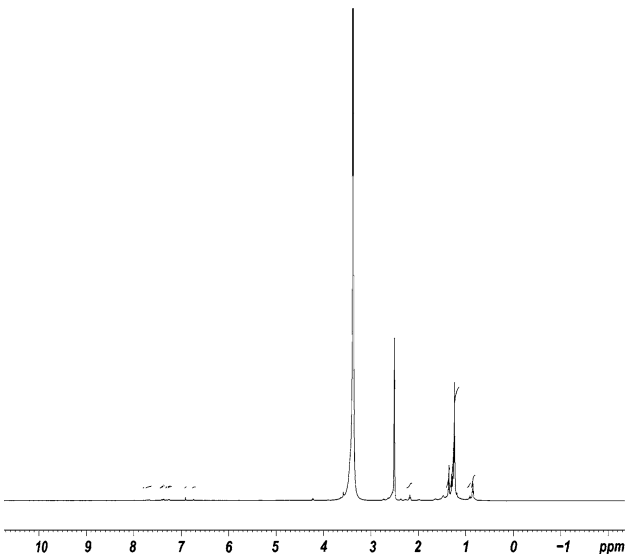

Fig. 4 ^{1}H nuclear magnetic resonance spectrum of purified siderophore produced by *V. harveyi* (VM1)

Binding properties of hydroxamate siderophore

A broad shift in λ^{max} (up to 52 nm) with different pH indicates the dihydroxamate nature of the siderophore produced. Absence of trihydroxamate and monohydroxamate nature was confirmed by this wide shift in λ^{max}. Electrophoretic mobility results for hydroxamate siderophores further confirm the dihydroxamate nature (Table 1).

Based on the stable or unstable nature of the colour of the ferrate siderophores at different pH values, the number of bonds that the ligand formed with metal ions was determined. In the present study the colour of the ferric hydroxamate remained red over a wide pH range (4–9), indicating hexadentate nature (Table 1). This is an important observation, and it is difficult to explain how the dihydroxamate siderophore can coordinate with a hexadentate ligand. Similar results of dihydroxamate siderophores with hexadentate ligands were reported earlier by Baakza et al. [1] in certain fungi. Winkelmann [41] reported that the iron-binding affinity of hexadentate siderophores is comparatively greater than that of other, bidentate types.

Photoreactivity

The effect of sunlight on purified siderophore was exhibited as a narrow shift in the UV–Visible spectrum (Fig. 5). Photolysis of iron-free hydroxamate siderophore was evident as a peak shift from 409 to 404 nm. Appearance of new peaks (320–400 nm) in the photoproduct is evident of photoreactivity. Photoreactive properties of ochrobactin and aerobactin produced by marine *Vibrio* species were earlier characterized [17, 20]. Barbeau et al. [3] reported a photolysis-induced ligand-to-metal charge-transfer band resulting in ligand oxidation and reduction of Fe(III) to Fe(II) state. These results suggest that photolysis of siderophores may contribute to iron cycling in ocean surface waters.

Table 1 Determination of mono-, di- or trihydroxamate nature of hydroxamate siderophore produced by the *Vibrio harveyi* (VM1) isolate

Isolate	λ^{max} (nm) of ferrate siderophore		λ^{max} (nm)	Colour of the ferrate siderophores in electrophoresis	Inference	Colour of the ferrate hydroxamate	Binding properties
	pH	λ^{max} (nm)					
VM1	4	443	52	Pink purple	Dihydroxamate	Red	Hexadentate
	5	495					
	6	452					
	7	478					
	8	448					
	9	451					

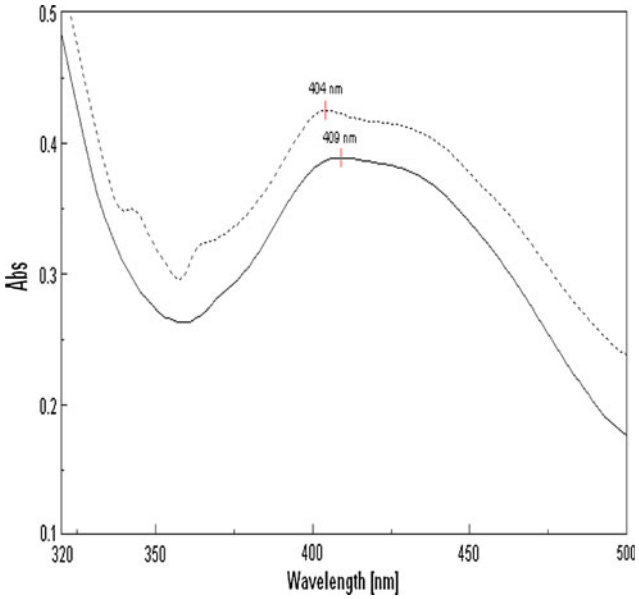

Fig. 5 UV-visible absorption spectra of hydroxamate siderophore, dark control and sunlight exposed

Biological activity

Although not tested in the producing strain, the purified hydroxamate showed good growth-promotion activity in biotests with *Staphylococcus aureus* and even more pronounced activity with *Aeromonas hydrophila*, confirming its function as a iron chelator. The negative result (absence of growth) with *E. coli* indicates the absence of a corresponding siderophore receptor and its inability to grow in low iron concentration (results not shown). The results of this study correspond well with earlier reports [9]. Stintzi et al. [34] reported that *Aeromonas hydrophila* possesses membrane receptors that are able to recognize and transport a range of siderophores as varied as catecholate, hydroxamate, etc., *Staphylococcus* strains are known to accept a variety of hydroxamate siderophores [33]. These results are in line with Dave et al. [8] in that the exogenous siderophore facilitates growth, promoting activity of other organisms under iron stress conditions.

Conclusions

This work establishes the presence of a siderophore-based iron-sequestration mechanism in *Vibrio* isolates. The ability to synthesize siderophores would undoubtedly be an advantage for survival, growth and pathogenicity of the isolates. The nature of the siderophores differs with species. However, characterization of siderophores based on the bacteria is not possible, because siderophores of varying nature have been produced by the same genus. This study intended not only to screen siderophore-producing marine *Vibrio* species but also to evaluate the influence of media on siderophore production and to isolate the best producer. In conclusion, we have shown that substrate choice is crucial for evaluation of siderophore production in microorganisms. Particularly, a substrate stimulating the organism must be used when the ecological role of siderophores is evaluated. This study reveals that the growth-promotion test is a fast and reliable bioassay for determining siderophore iron-chelating behaviour. Different spectral analyses used in this study reveal more structural information about the siderophore produced, which could lead to better understanding of how it mediates Fe transfer. Mass-spectral characterization is in progress for structure determination.

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