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ISOLATION AND CHARACTERIZATION OF INDIGENOUS LUMINESCENT MARINE BACTERIA FROM KARACHI COAST

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ABSTRACT

A luminescent bacterial strain was isolated from sea water samples from the shore of the Arabian Sea, Pakistan. The isolate was identified as Vibrio harveyi species upon biochemical and 16SrRNA gene analysis and coded N6. The isolated strain was subjected to physical and genetic characterization. Upon study of the genetic markers present in the isolate, it was observed that the bacterium tolerated up to 7% of Sodium chloride in simple nutrient broth medium. Unlike commonly reported luminescent bacteria, the indigenous isolate N6 showed optimum growth at 37ºC. Resistance towards low concentrations of Cadmium chloride and Copper sulfate was also recorded in N6. Of the various antibiotics screened for resistance, N6 was highly resistant to Ampicillin. No plasmid DNA was observed in the strain. The best carbon source supplemented in minimal medium was determined to be 0.2% of gluconate which gave the best growth but luminescence was not achieved on minimal medium in presence of carbon sources like glycerol, gluconate, glucose, fructose, sucrose, starch nannitol, lactose, galactose and maltose. Presence of the lux operon was determined by performing PCR for the luxAB genes, the PCR product obtained was sequenced to reveal major similarities with previously reported luxAB genes.

Keywords: *16S rRNA PCR identification, Characterization, Growth optimization, Isolation, Vibrio harveyi, luxAB genes PCR.*

INRODUCTION

Luminous bacteria are the most widely distributed light-emitting organisms, most of which are found in sea water and the remainder living in the terrestrial or fresh water environment. These luminescent bacteria are usually found in symbiotic association with a host organism but are also capable of living free. The most common habitats are as free-living species in the ocean, as saprophytes growing on dead fish or meat, as gut symbionts in the digestive tracts of marine fish, as parasites in crustacean and insects and as light organ symbionts in teleost fishes and squid (Hastings, 1986). These bacteria are all Gram negative rods and can function as facultative anaerobes (Baumann et al, 1983 & Nealson and Hastings, 1979)). Almost all luminous bacteria have been classified into three genera *Vibrio, Photobacterium* and *Xenorhabdus,* with most of the species being marine in nature (Baumann et al, 1983 & Campbell, 1989). The light emitting bacteria that have been investigated in most detail are *Vibrio harveyi, V. fischeri, Photobacterium phosphoreum, P. leognathi and Xenorhabdus luminescens.* The *Vibrio harveyi* strains have not yet been found as symbionts, although they are readily isolated from varying marine habitats (Meighen, 1991), these species may also be found on the surface of marine animals or in their gut (Baumann et al, 1973 & Ruby and Morin, 1979).

The luminescence of these bacteria is attributed to the presence of an intricately working group of genes of the lux operon basically containing the genes luxICDABEG (Meighen, 1991). This luminescent system is self contained as the substrate and the enzyme for the luminescent reaction is made by the lux system itself. The enzyme working in this reaction is known as luciferase which is a heterodimer comprising of an α and a β subunit, the substrate is a long chain fatty aldehyde specific for each specie of the luminescent bacterium. In case of *Vibrio harveyi*, this aldehyde is either a

nonanal or a decanal. The lux system is induced by an inducer molecule which is also produced by the lux gene cluster itself. These inducer molecules are homoserine lactones and are specific for each bacterial specie as well. *Vibrio harveyi* lux operon is induced by the inducer α-hydroxybutrylhomoserine lactone (Meighen, 1991). The luminescent reaction involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue-green light.

A number of strains of luminescent bacteria have been isolated from ocean and sea samples from around the world. Both temperate and tropical sea environments support the inhabitance of marine luminous bacteria (Ruby and Lee, 1998). Abiotic factors such as patterns of temperature, salinity, nutrient concentration or solar irradiation (O'Brien and Sizemore, 1979; Ruby and Nealson, 1978; Shilo and Yetinson, 1979; Yetinson ans Shilo, 1979) play a major part in determining the presence of luminous bacteria in a certain marine environment. Luminous bacteria have previously been isolated from coastal marine samples of Hawaii, Alaska, Manzanita OR, pacific ocean, Mexico, Uruguay, north Atlantic, Oslo Harbor, Norway, France, Israel, South Africa,Philippines, Taiwan and Japan (Urbanczyk, 2008). The present study reports the isolation of luminescent bacterial isolate of *Vibrio harveyi* from the Arabian Sea shore and general characterization of the bacterial isolate was performed to identify the genetic markers present on the bacteria.

MATERIALS AND METHODS

Isolation and identification of bacterial isolates

Sea water samples were collected from a jetty located at the Boating Basin area of Karachi. Water samples were collected by submerging autoclaved dark bottles under water and then opening their caps. The bottles were sealed under water and then kept in dark at 4ºC until processed for isolation of bacterial strains.

300µL of the water samples were spread onto 4% agar plates of 25mL LSW-70 (Ast and Dunlap, 2004) and incubated overnight at 25°C. Agar percentage was kept high initially i.e. at 4% so as to restrict overgrowth of any spreading forms of non-luminescent bacteria present in the sample. The brightest bacterial colonies were selected upon observation in a dark TLC viewer. The isolate was purified by re streaking onto 1.8% agar plates of LSW70 and gram stained for cellular characterization. Purified bacterial culture was then coded N6 and employed for further experimentation.

The isolate was also checked for motility under dark field microscope by placing a drop of overnight culture onto a glass slide. Motility was observed in a dark field microscope at 40X magnification.

Biochemical identification

N6 was sent for biochemical identification and was identified using an indigenous system, QTS-24, akin to the API system of bacterial identification.

16SrRNA Gene Analysis

Genomic DNA of the marine isolate was isolated using genomic DNA isolation kit (GENTRA, USA) and 16SrRNA gene analysis was performed. The genomic DNA was used as template DNA in a touchdown PCR reaction (Thermal cycler: Applied Biosystems, GeneAmplification system 2400) using the universal primers **16S-5F** 5'- TGGAGAGTTTGATCCTGGCTCAG -3' and **16S-531** 5'-TACCGCGGCTGCTGGCAC -3', which amplify a sequence of 550 bps (approx.). The reaction consisted of 37 cycles i.e. 94ºC for 3min. (1 cycle) 94ºC for 1min. 62ºC for 30sec, 72ºC for 3min. (2 cycles) and then each following cycle after every two cycles had a consecutive decrease of 1ºC in the

primer annealing step upto 50ºC. A final extension time was given for 5 min at 72ºC. (Scott, 2002). GeneAmp® PCR Core Reagents were used for the PCR reaction mixture which composed of: 5µL of 10X PCR buffer, 1µL dNTP mix (200µ*M*), 0.5µL AmpliTaq® DNA polymerase, 2µL of each primer (20pmol), 1µL DNA Template and 38.5µL sterilized distilled water, bringing the total volume up to 50µL.

PCR products were purified using QIAquick PCR purification kit (Qiagen, UK), purified DNA was air dried and commercially sequenced by Microsynth AG, Switzerland. Sequence data obtained were analyzed using BLAST algorithm (http://www.ncbi.nlmnih.gov/blast/Blast.cgi).

Growth curve of N6

Growth curve experiment was performed in LSW 70 medium. 100mL of the LSW 70 broth was inoculated with an overnight grown culture of N6, such that the starter $O.D_{600}$ was 0.075. Observations for growth O.D was taken using a spectrophotometer (Cam spec M302) at 600nm of absorbance after every 15 minutes till the log phase was achieved, after which readings were taken every 30 minutes. Zero reading was calibrated with un-inoculated LSW70 broth. Growth curve was plotted as $O.D_{600}$ readings against time and different phases of growth were hence determined.

Growth optimization in minimal medium

Minimal medium used for the study was ASW (Artificial Sea Water) (Macleod, 1968) which was supplemented with 0.3mM K_2HPO_4 (as phosphate source), 15mM ammonium chloride and 1mL/liter of a 20mg/mL filter sterilized solution of ferric ammonium citrate (as source of iron) (Dunlap, personal communications). Solutions for NH_4Cl and K_2HPO_4 were made as 1M stock solutions and sterilized by autoclaving at 121°C for 20 minutes. To obtain the optimum growth and luminescence in minimal culture, different carbon sources were tested like glycerol, gluconate, glucose, fructose, sucrose, starch, mannitol, lactose, galactose, and maltose. The sugar solutions were made as 10% stock solutions and sterilized by autoclaving at 115°C for 10 minutes. The experiment was carried out in broth as well as solid phase of the medium; 2% agar plates of ASW were prepared with 0.2% of each of the carbon source except glycerol which was added at a concentration of 3mL/Liter.100µL of overnight culture of N6 grown in LSW70 was inoculated and incubation was given at 25ºC with shaking at 120rpm for the broth cultures. Results for growth and luminescence were checked after 24Hrs of incubation.

Also, to induce luminescence in the minimal medium 10% v/v of conditioned medium was also added in the medium (Dunlap et al, 1995). 50mL of spent medium was extracted out of an overnight ASW culture by centrifuging the culture in alcohol sterilized 100mL centrifuge tubes in a cooling ultra centrifuge for 15 minutes at 4°C at 7000rpm. The supernatant (spent medium) was separated and filter sterilized using a 0.2µm Millipore filter. 0.02% of the amino acid Arginine was also added to the ASW broth medium to aid in luminescence induction.100µL overnight culture was inoculated and then incubated overnight at 25°C with shaking at 120rpm in a cooling incubator.

Maximum tolerable concentration for Sodium Chloride

Maximum tolerable concentration for Sodium Chloride was checked in nutrient agar. The range in the medium was checked from 0% to 15% of NaCl. Overnight cultures of the N6 was streaked onto the NaCl test plates and incubated overnight at room temperature (25°C).

Maximum tolerable concentration for antibiotics

Maximum tolerable concentration of antibiotics for the luminescent isolate was checked in 1% LB. The antibiotics tested for maximum tolerable concentration were Tetracycline, Streptomycin, Kanamycin and Ampicillin. The range of concentrations started from 25µgm/mL to 900µgm/mL. N6

was streaked on the different antibiotic plates and incubated at 25.7°C overnight. Results for growth and luminescence were checked after 24Hrs of incubation.

Maximum tolerable concentration for heavy metal salts

Maximum tolerable concentration for selected heavy metal salts, i.e. Cadmium chloride, Copper sulfate and Hexavalent chromium, was checked on 1.8% agar plates of LSW70. Growth in presence of these heavy metal salts was also checked in minimal medium as well, the selected medium for this purpose was Artificial Sea Water (ASW) supplemented with 0.8% gluconate. 100µL of overnight cultures of N6 was inoculated in the ASW broths in 50mL flasks with 0.5mM of each of the selected heavy metal salt added individually. The flasks were incubated at 25°C with continuous shaking at 120rpm. The isolates were also streaked onto 1.8% agar plates of LSW70 with heavy metal. The concentrations of each heavy metal salt checked were 0.5mM, 1.0mM, 1.5mM and 2.0mM. These plates were incubated at 25.7°C in a cooling incubator. Results for growth and luminescence were checked after 24Hrs and 72Hrs of incubation.

Plasmid isolation

Plasmid isolation was attempted using the method described by Brinboim and Doly (Brinboim and Doly, 1979) and also by using the plasmid isolation kit by QIAGEN. Plasmid isolation was performed with an overnight culture grown in LSW70 of which 1.5mL was pelleted at 13000g. The plasmid DNA was visualized by gel electrophoresis and staining the gel with ethidium bromide.

LuxAB PCR amplification

Partial gene amplification of the *luxAB* genes was performed to determine whether the genetic element responsible for luminescence in N6 is similar to the reported *lux* system. The isolated genomic DNA was used as template DNA in a PCR reaction (Thermal cycler: Eppendorf AG thermal cycler, No. 5345) using the primers **LuxAB-F** 5'- CGG GAT CCA ACA AAT AAG GAA ATG TTA TG -3' and **LuxAB-R** 5'- CCA GAT CTT CCA TAT AAA TGC CTC TAT TAG -3', which amplify a sequence of 2179 bps (approx.). The reaction consisted of 33 cycles i.e. 94ºC for 3min. (1 cycle) 94ºC for 1min. 55ºC for 30sec, 72ºC for 3min. (30 cycles). A final extension time was given for 5 min at 72ºC (1 cycle) and a hold of 4.0ºC. GeneAmp® PCR Core Reagents were used for the PCR reaction mixture which composed of: 5µL of 10X PCR buffer, 1µL dNTP mix (200µ*M*), 0.5µL AmpliTaq® DNA polymerase, 2µL of each primer (20pmol), 1µL DNA Template and 38.5µL sterilized distilled water, bringing the total volume up to 5Germany), purified DNA was air dried and commercially sequenced at Microsynth AG, Switzerland. Sequence data obtained were analyzed by using BLAST algorithm (http://www.ncbi.nlmnih.gov/blast/Blast.cgi).

RESULTS AND DISCUSSION

Isolation of bacterial isolates

Bright luminescent colonies were observed, in a dark room, spread over the 4% agar plates of LSW70 after 24 hours of incubation (figure 2). The luminescent bacteria give off a bright blue-green light that is easily observed through dark adjusted unaided eyes. Once the choice bacterial colonies were selected and isolated, the agar concentration was brought down to 2.5% and eventually 1.8% with consecutive culturing and purification. Eight of the brightest colonies were selected and streaked onto fresh LSW70 plates for purification, however only five of which remained luminescent after re streaking of which only one strain was selected for study and coded N6. Such loss of luminescence has been reported before and it appears with high frequency in enriched cultures of continuously cultured luminescent isolates and is termed as dark mutants (Keynan and Hasting, 1961). N6 upon study of cellular morphology revealed scattered short cocobacilli that give a Gram negative reaction.

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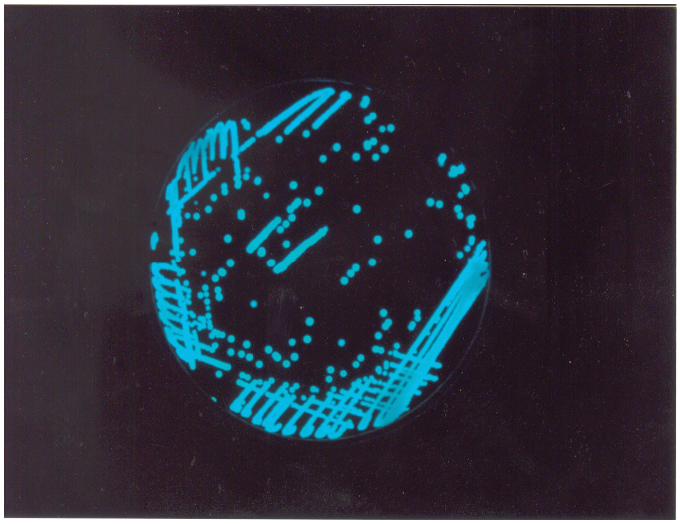

Figure 2: Streaked plate of N6 on LSW70 as observed in dark

N6 showed motility i.e. darting movement characteristic of *Vibrio*s when observed on a wet mount. On solid medium it formed small yellowish colonies that were luminescent when observed in dark. The isolate did not give a highly motile growth on the solid medium and hence did not tend to spread on the agar plate.

Broth culture of the isolate gave a pellicle formation when grown at 25°C with slight shaking in a test tube, this pellicle gave luminescence when observed in dark with dark adjusted eyes. The broth was observed to turn slightly yellow after 24Hrs of incubation. Optimum temperature for growth was observed to be 37ºC unlike the lower range of temperatures, 20-25ºC reported for other luminescent strains. This may be due to the fact that the sea water temperature of the Arabian Sea is higher as compared to the temperature of waters from where previous isolations have been reported (Urbanczyk et al, 2008).

Upon biochemical identification, all the fermentation reactions were identical with *Vibrio cholerae*. Parameters like growth at 4°C and 40°C, luminescence and the ability to ferment the sugars D-Gluconate and Lactate (Boettcher and Ruby, 1990), distinguished N6 from other luminescent *Vibrio* and identified it as *Vibrio harveyi.* A few of the biochemical test results are given in table 1.

Table 1: Biochemical test results for Luminescent *Vibrio harveyi* strain N6

Strain code	ONPG	Citrate	Malonate	H_2S	Urea	Indole	VP	Glucose/NO_3	Maltose	Sucrose	Mannitol	Arabinose	Rhamnose	Motility
N6	+	-	-	-	-	+	-	+/+	+	+	+	-	-	+

The partial 16S rRNA gene sequence data was analyzed using NCBI BLAST algorithm search and the *Vibrio* isolate N6 (Accession # DQ166246) was identified as *Vibrio harveyi* specie.

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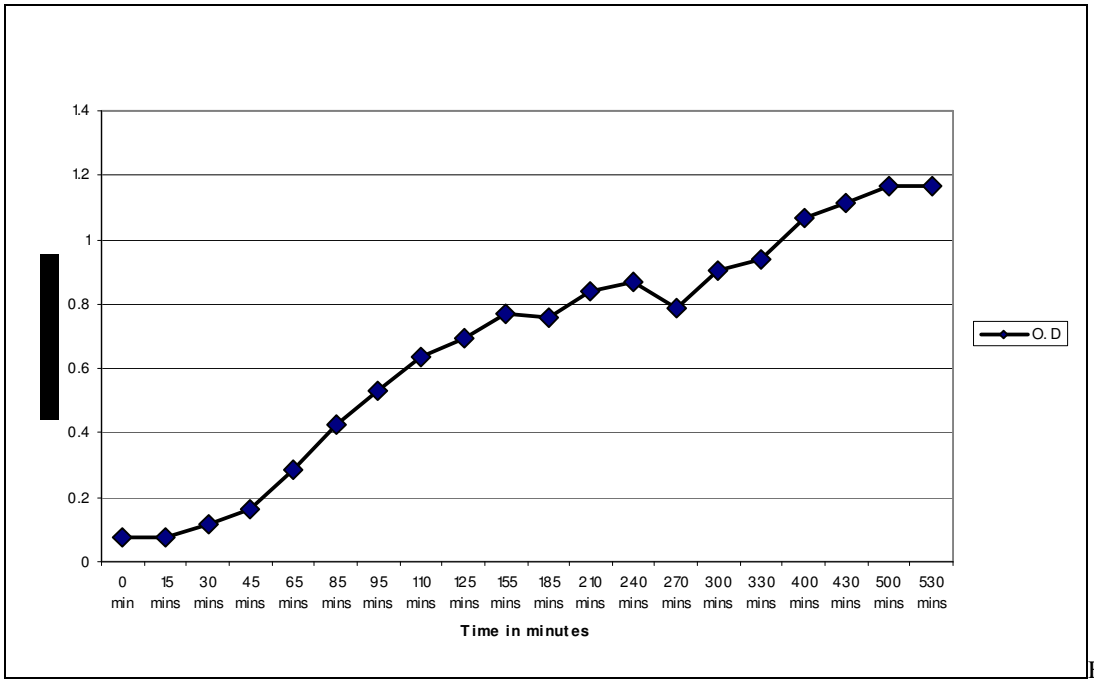
Performance of growth curve

Growth curve experiments revealed that N6 is a fast growing bacterial isolate that reached its log phase after 30 minutes of incubation (figure 1) at 25.7°C with continuous shaking of 120rpm. The log phase continues up to 7.0Hrs after which the stationary phase seems to start (figure 1).



Figu

Figure 1: Growth curve of N6 in LSW 70.

Growth optimization in minimal medium

Growth of N6 was optimized for minimal medium so that the luminescent bacteria may be employed in studies with heavy metals. As enriched and complex media chelate the heavy metals in medium, making them less bioavailable hence a minimal medium is choice for such studies. Upon ASW agar plates supplemented with various carbon sources, N6 exhibited exceptionally good growth in presence of 0.2% gluconate with a comparatively slightly less growth in presence of 3mL/Liter of glycerol, but with this growth no visible luminescence has been achieved.. The rest of the carbon supplements did not give an exceptional growth under the conditions tested.

Maximum tolerable concentration for Sodium Chloride

After 24Hrs of incubation at room temperature, N6 showed good growth and maximum luminescence as compared to the other test plates. There was no growth observed with 0% NaCl, indicating the requirement of the salt for growth. This has already been established previously that *Vibrios* have specific growth requirements for sodium ion (Nealson and Hastings, 1979). After 48Hrs of incubation the nutrient agar plates with 1% and 3% NaCl showed the maximum and the most persistent luminescence as compared to other salt concentrations. At this point however the 2% NaCl plate gave diminished luminescence. It is hence suggested that at 2% NaCl concentration, N6 grows faster but maximum luminescence is obtained with 3% NaCl in the medium. N6 is also able to tolerate up to

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7% of NaCl in their culture medium (table 3). Beyond the concentration of 4% NaCl, N6 gave very low luminescence. It is thought that as luminescence is a character that is expressed under enriched nutrient conditions, hence in presence of high NaCl concentrations that pose as a stress factor, luminescence is diminished.

Table 3: 24Hrs results for the maximum tolerable concentration of Sodium Chloride and the observance of luminescence in presence of different salt concentrations in nutrient agar.

	N6
Control 0% NaCl	-
1%	+++*
2%	+++*
3%	+++*
4%	+++*
5%	+++*
6%	++*
7%	++*
8%	-
9%	-
10%	-
11%	-
12%	-
13%	-
14%	-
15%	-

+++ High growth

++ Weak growth

* Luminescence, observed with naked eye.

Maximum tolerable concentration for antibiotics

N6 showed a very high resistance towards Ampicillin, the tolerable concentration going well above 900µgm/mL of the medium. It was sensitive to Tetracycline and fairly resistant to Kanamycin and Streptomycin. Luminescence in presence of the antibiotics was also checked and it was observed that as the stress of the antibiotic in the medium increased, luminescence decreased. The results are given in table 4. N6 showed its optimum luminescence in presence of Ampicillin at a concentration of up to 900µgm/mL, while luminescence disappeared after a concentration of 25µgm/mL of Kanamycin. No luminescence was observed in presence of Streptomycin. The expression or absence of luminescence in presence of the antibiotics indicates the level of toxicity of each antibiotic towards the luminescent isolate.

Table 4: Maximum tolerable concentration for selected antibiotics in 1% NaCl Luria agar medium.

	Ampicillin	Tetracycline	Streptomycin	Kanamycin
N6	>900□gm/mL	-	50□gm/mL	75□gm/mL

Maximum tolerable concentration for heavy metal salts

After 24Hrs of incubation in ASW broth medium N6 showed good growth in presence of 0.5mM of Cadmium chloride, while growth was observed in 0.5mM of Copper sulfate after 72Hrs of incubation. No luminescence was observed in any of the minimal broths including the control flasks as well.

In case of the heavy metal salts supplemented in the enriched LSW-70 medium, growth was only observed for the Cadmium chloride and Copper sulfate supplemented plates. Luminescence was observed in the culture but it decreased with the increasing concentration of the heavy metal salts. After a lapse of 72Hrs luminescence in presence of all the concentrations of Cadmium chloride and Copper sulfate disappeared. This probably happened as the toxicity of the heavy metal salts increased for the growing culture i.e. toxicity effect increased with longer exposure of the heavy metals to the dividing bacterial isolate. The observation that luminescence decreases with the increasing concentration of cadmium chloride can be employed as an assay for the heavy metal in environmental samples and such a technology has been successfully adopted and marketed as Microtox®, which is based on the use of the wild type luminescent bacterium *Vibrio fischeri* (Johnson and La Rossa, 1998 & Qureshi et al, 1998) .

Plasmid isolation

No plasmid DNA was found to be present in the luminescent isolate N6.

LuxAB PCR amplification

The DNA sequence obtained by *luxAB* PCR amplification was analyzed using NCBI BLAST algorithm search. The sequence data of N6 (Accession # EF141075) showed high homology with the luxAB gene sequence data in the gene bank giving up to 98% homology with reported *luxAB* gene sequences of other *Vibrio harveyi* strains in the database.

CONCLUSIONS

The isolation of the luminescent *Vibrio harveyi* strain N6 from the coast of Karachi is an indigenous discovery. Such marine luminescent bacteria have never been reported from this region and hence hold the promise for the development of indigenous biotechnological tools like heavy metal biosensors. The isolation of N6 from Karachi coast also indicates towards the widespread of this specie and its adaptability in all kinds of environments ranging from icy cold oceans to temperate seas like the Arabian Sea.

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