Isolation, identification and characterization of new luminous bacteria from Chah Bahar Port, southern marine habitat of Iran

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Abstract

Coastal region of Chah Bahar port, Sea of Oman, was screened for the presence of bioluminescence bacteria for the first time. Water samples were taken from surface and subsurface layers and immediately spread on nutrient seawater complete (SWC) agar. Luminous colonies were observed after an overnight incubation at 25°C. Among twenty luminous isolates, four of them were selected for preliminary bacterial identification based on morphological and physiological characteristics. 16S rRNA genes of selected bacteria were then sequenced in order to be submitted in GeneBank database as new strains and performing phylogenetic analysis. Four different submitted bacterial strains are as follow, *Vibrio* sp. Persian 1, *Vibrio* sp. Persian 2, *Vibrio* sp. Persian 3, and *Vibrio* sp. Persian 4 with accession numbers of KC505639, KC765088, KC765089, and KC896417, respectively. Light emission of isolated luminous bacteria was measured using luminometer. *Vibrio* sp. Persian 1was found as the best light emitter with counts per second/OD 600 nm equal to 10×10^{6} RLU/Sec/OD. Isolated *Vibrio* species were tested for their ability to form biofilm. *Vibrio* sp. Persian 3 showed weak ability to produce biofilm while other species were considered as moderate biofilm producers.

Keywords: Bioluminescence,16S rRNA genes, Vibrio, Luminometer, Biofilm.

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Introduction

Bioluminescence is the emission of light by living organisms and occurs in an array of organisms including fish, insects, jellyfish, and bacteria. The function of bioluminescence may vary from one organism to the other, such as defense against predators, predation or communication with their mates. Bioluminescent bacteria are the most abundant and widely distributed of all light-emitting organisms, occupying a wide variety of ecological niches (fish light organs, mammalian gut, nematode gut) and habitats (marine, freshwater, terrestrial, and symbiotic within a host). While most species of luminescent bacteria are capable of living free, the majority are found in nature associated in symbiosis with host organisms. Currently, only four genera of bacteria are known to naturally bioluminescence: *Vibrio*, *Photobacterium*, *Shewanella*, and *Photorhabdus* (Hastings *et al*., 1985; Meighen, 1994; Peat and Adams, 2008). Presence of at least five genes arranged in an operon: luxCDABE is essential for bacterial bioluminescence and light emission reaction in bacteria is catalyzed by luciferase, encode by luxAB, which is a heterodimeric protein composed of α (40-kDa) and β (36-kDa) subunits. Bacterial luciferase mediates the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde (RCHO) by molecular oxygen (O_2) to produce blue-green light according to the following reaction:

$$FMNH_2 + O_2 + RCHO \longrightarrow FMN + H_2O + RCOOH + Light\ (490nm)$$

Although light emission is the first characteristic of bioluminescence, there are several investigations which showed the presence of lux operon, responsible for light production, in some bacteria despite the absence of their obvious visual phenotype. These non-visibly luminous strains, called dark mutants, are as the result of mutations in lux operon (Ramaiah *et al*., 2000; O'Grady and Wimpee, 2008). As expression of luminescence in many bacteria is highly depends on cell density, single bacteria living free in the ocean would not be expected to emit light. Luminescence will thus only be observed for bacteria growing in a confined environment such as in the light organs of fish or squid or in colonies on a solid nutrient source (Meighen, 1993). Since southern marine environments of Iran have never been screened for bioluminescent bacteria, in this study we focused on water sampling from Sea of Oman and isolating possible new luminous strains. Bacterial identification of luminous isolates based on molecular techniques such as 16S rRNA gene sequencing, ARDRA, and AFLP have been reported in several investigations (Benediktsdottir *et al*., 2000; Kita-Tsukamoto *et al*., 2006). Furthermore, identification based on conventional phenotypic characteristics may be affected by biochemical activities of each bacterial strain; so morphological, biochemical, and molecular characterizations were performed to confirm the identification of luminous isolates and then submitting them in GeneBank Database. Since biofilm

formation is a mechanism which depends on regulatory process such as quorum sensing (Yildiz and Visick, 2009) and it is believed to play an important role in the virulence of aquatic pathogens (Bjelland *et al*., 2012), biofilm formation as well as light emission by the isolated bacteria were tested in order to achieve higher order of isolates characterization.

Materials and Methods

Sampling, collection, and isolation of luminous bacteria

Two samples were collected by divers from subsurface (1 m) and bottom (15 m) of seawater within the 1 km of shore, in Chah Bahar Port. The Port of Chah Bahar is located in the south-east of Iran, north-west of Indian Ocean, and north-east of Oman Sea at 25°17′28″N 60°38′15″E 25.29111°N 60.6375°E. The location of sampling from subsurface and bottom layers was 50 m apart from each other. Water samples were collected by submerging autoclaved bottles under water and then opening their caps. The bottles were sealed under water to keep the exact microbial population of selected water layers. Two hundred µl of the water samples were spread onto nutrient seawater complete (SWC) agar plates containing 3 ml of glycerol, 1 g of yeast extract, 3 g of peptone, and 15 g of agar (Merck, Germany) per liter of 75% seawater (O'Grady and Wimpee, 2008), and then incubating overnight at room temperature. The appearance of luminescent colonies was observed in a dark room after dark adapting for 5 min. The brightest bacterial colonies were selected for more characterization and these isolates were restreaked onto SWC agar plates in order to gain purified colonies. The resulting isolated colonies were maintained at room temperature and subcultured at four-week intervals. Long-term storage was at -196 °C, liquid nitrogen, in SWC broth supplemented with 10% glycerol.

Bacterial identification

Bacterial characterization was preliminary performed based on cellular and microscopic morphology, and biochemical tests. Gram staining was done as a common differentiating technique. Biochemical tests used in this study were as follow: carbohydrate fermentation includes galactose, maltose, manitol, sucrose, and arabinose, Simmons' Citrate, MRVP (Methyl Red, Voges-Proskauer), indole, nitrate reduction, gelatinase, urease, oxidase, catalase, and TSI (Triple sugar iron agar). Capability of growing on TCBS (Thiosulfate-citrate-bile salts-sucrose) agar medium was also tested to meet the nutritional requirements of *Vibrio* spp., as an important genus of light producing bacteria. All biochemical tests were done by incubating bacteria at room temperature. As a complement to confirm the identification of luminous strains, molecular analysis based on 16S rRNA gene sequencing was carried out.

Extraction of genomic DNA and PCR

Genomic DNAs were extracted using Phenol-Chloroform extraction method as described by Sambrook *et al*., (1989).Viability and presence of extracted genomic DNA was confirmed by 1% agarose gel electrophoresis. The final concentration of PCR reagents in each 25µl reaction was as follow: 200 µM dNTPs, 1X

PCR buffer, 1.5Mm $MgCl_2$, 1U Taq polymerase (Fermentas, Germany) and 1.5 µM of each forward and reverse primers (Sigma Co.). Adding sterilized distilled water brought the total volume up to 25 µL. Universal primers used had nucleotide sequence as bellow and amplify a sequence of approximately 1400 bps:

Forward primer:
5'- AGAGTTTGATCATGGC-3'
Reverse primer:
5'-TACCTTGTTACGACTT-3'

To increase specificity and sensitivity of bacterial 16S rDNA amplification, touchdown PCR was performed as following condition (Don *et al*., 1991): 5 min of initial denaturation at 94°C, followed by 10 cycles of 94ºC for 1min, 60ºC for 1min, 72ºC for 1min and then each following cycle had a consecutive decrease of 1ºC in the primer annealing step up to 50ºC. Twenty cycles were added to support the exact amplification of desired template. With the exception of a constant annealing temperature of 55ºC, all the steps were the same as the first 10 cycles. The PCR was terminated after a final extension of 7 min at 72°C. PCR products were sent to SQ lab Co. (Germany) in order to be sequenced using Sanger method (Sanger *et al*., 1977). By receiving the results, the full sequenced (1,400 bp) of the 16S rRNA genes were compiled with Gene Runner software version 3.05 and then they were deposited in GenBank and aligned with the 16S rDNA sequences available in nucleotide database in NCBI, (National Center for Biotechnology Information, Available at: http://www.ncbi.nlm.nih.gov), using BLAST software, (Basic Local Alignment Search Tool). Confirmed isolated bacteria were submitted as new indigenous species in GeneBank database via Bankit submission tool.

Light emission measurement

The cells were incubated in the SWC broth with a rotary shaker (120 rpm) at 25ºC for an overnight and the light output of each isolated strain was determined with a luminometer (Berthold Detection system, Germany) and was reported as Relative Light Units (RLU/Second) divided by the optical density at 600 nm (OD_{600}) of the culture (Shao and Hor, 2001).

Biofilm assay

Biofilm formation was assayed by the modified method of Vikram *et al*. (2010). Briefly, bacterial colonies were grown overnight at 25ºC in SWC broth. The bacterial culture were then diluted 1:100 in a new SWC medium and 150 µl of this dilution was used to inoculate the sterile flat-bottomed 96-well polystyrene microtiter plates. The culture was placed in polystyrene 96-well plates and incubated at 25ºC for 24 h without shaking. Thereafter, wells were gently washed three times with 200 µl of PBS and each well was exposed to 100 µl of 99% methanol for 15 min for fixation. The solutions were then removed and the plate was air-dried. In the next step, 150 µl of crystal violet 1% was added to all wells for 20 min. After removing the dye, the dye associated with biofilm was dissolved with 150 µl 33% acetic acid, and the OD of each well was measured at 590 nm using a microtiter plate reader. All the assays were done in triplicate and uninoculated SWC wells were used as negative control to determine background

OD. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. The isolates were classified into the four following categories based on the optical density (Stepanovic´ *et al*., 2007): non-biofilm producers (OD test<ODc), weak biofilm producers (ODc<OD<2×ODc), moderate biofilm producers (2×ODc<OD < 4×ODc), and strong biofilm producers (4× ODc<OD).

Results

Bacterial identification

A total of twenty bright colonies were isolated in which four of the brightest colonies were selected for more experiments. One of the strains was isolates from the subsurface layer of water, designated as 4, and the rest were found from the bottom sampling location, designated as 1, 2, and 3. First step of bacterial characterization by gram staining revealed that all the isolates were gram negative rods, an expected finding provided by others (Baumann *et al*., 1983).The results of biochemical tests are shown in Table 1. Since the use of biochemical identification as the sole method could not accurately determine genotypic features of luminous isolates, species identification of these isolates was performed by 16S rRNA gene sequencing analysis. The BLAST search of 16S rRNA gene sequences of isolated bacteria against sequences in nucleotide database revealed that all luminous species belong to genus Vibrio. Bacterial isolates 1, 2, 3, and 4 were submitted in GenBank database as Vibrio sp. Persian 1 with the accession number of KC505639, *Vibrio* sp. Persian 2 with the accession number of KC765088, *Vibrio* sp. Persian 3 with the accession number ofKC765089, and *Vibrio* sp. Persian 4 with the accession number of KC896417, respectively.

Table1: Physiological characteristics of isolated luminous bacteria. Bacterial isolates: 1, *Vibrio* sp. Persian 1; 2, *Vibrio* sp. Persian 2; 3, *Vibrio* sp. Persian 3; 4, *Vibrio* sp. Persian 4.

Strains / Tests	1	2	3	4
Galactose	+	+	+	+
Maltose	+	+	+	+
Sucrose	-	-	+	-
Manitol	+	+	+	+
Arabinose	-	-	-	-
Simmon Citrate	-	-	-	-
MR	+	+	+	+
VP	-	-	-	-
Indole	-	-	-	-
Nitrate	+	+	+	+
Gelatinase	+	-	-	+
Urease	+	+	+	+

Table 1 continued:

TSI	Alkaline/Acid*	Alkaline/Acid	Alkaline/Acid	Alkaline/Acid
Oxidase	+	+	+	+
Catalase	+	+	+	+
Growth on TCBS Agar	+ with green colonies	+ with green colonies	+ with yellow colonies	+ with green colonies

* Alkaline /Acid exhibits capability of glucose fermentation without any gas production

Light emission measurement

Measurement of the amount of light produced by isolated bacteria, shown in Fig. 1, revealed that isolated bacteria belong to the same genus, *Vibrio*, could represent different bioluminescence activity. *Vibrio* sp. Persian 1 was the best light producer followed by *Vibrio* sp. Persian 2 and 4, which displayed the same bioluminescence phenotypes, around 7×10^6 RLU/Sec/OD. Among 4 isolated bacteria *Vibrio* sp. Persian 3 could not emit light as much as the others.

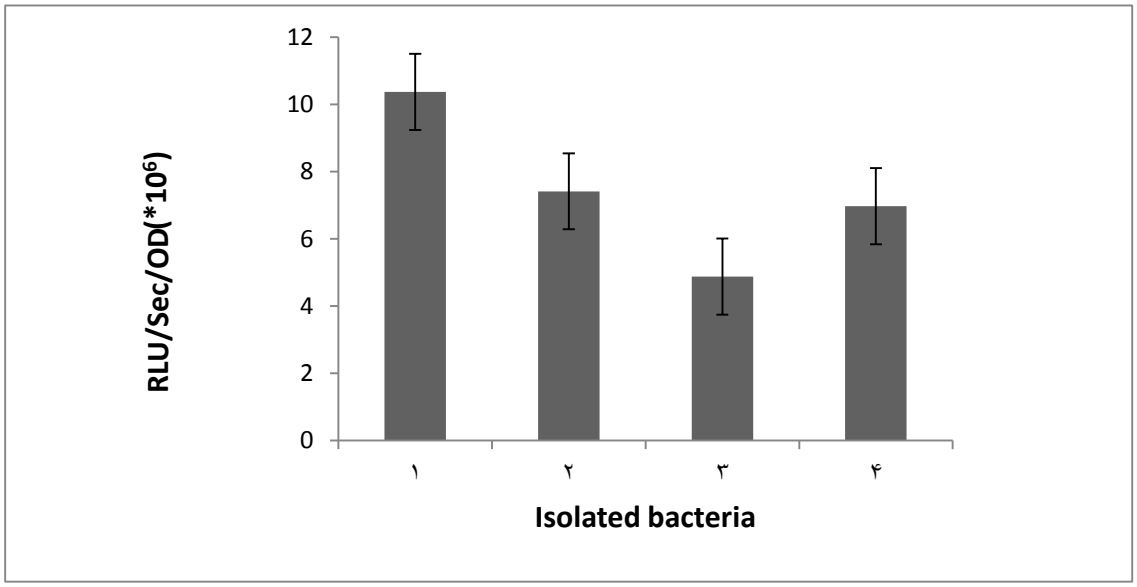

Figure 1: Light output measurement of each bacterial isolate as Relative Light Units i.e. counts per second divided by optical density at 600 nm (OD 600 nm) of the culture. Error bars represent standard errors. Bacterial isolates: 1, *Vibrio* sp. Persian 1; 2, *Vibrio* sp. Persian 2; 3, *Vibrio* sp. Persian 3; 4, *Vibrio* sp. Persian 4.

Biofilm formation

Amount of biofilm production based on OD measurement at 590 nm is shown in Fig.2. According to these, *Vibrio* sp. Persian 1and *Vibrio* sp. Persian 4, with similar OD, *Vibrio* sp. Persian 2 and *Vibrio* sp. Persian 3, with different capacity for biofilm production, were able to form biofilm. Furthermore, statistical analysis revealed that *Vibrio* sp. Persian 3 was a weak biofilm producer while the remaining species were considered as moderate biofilm producers.

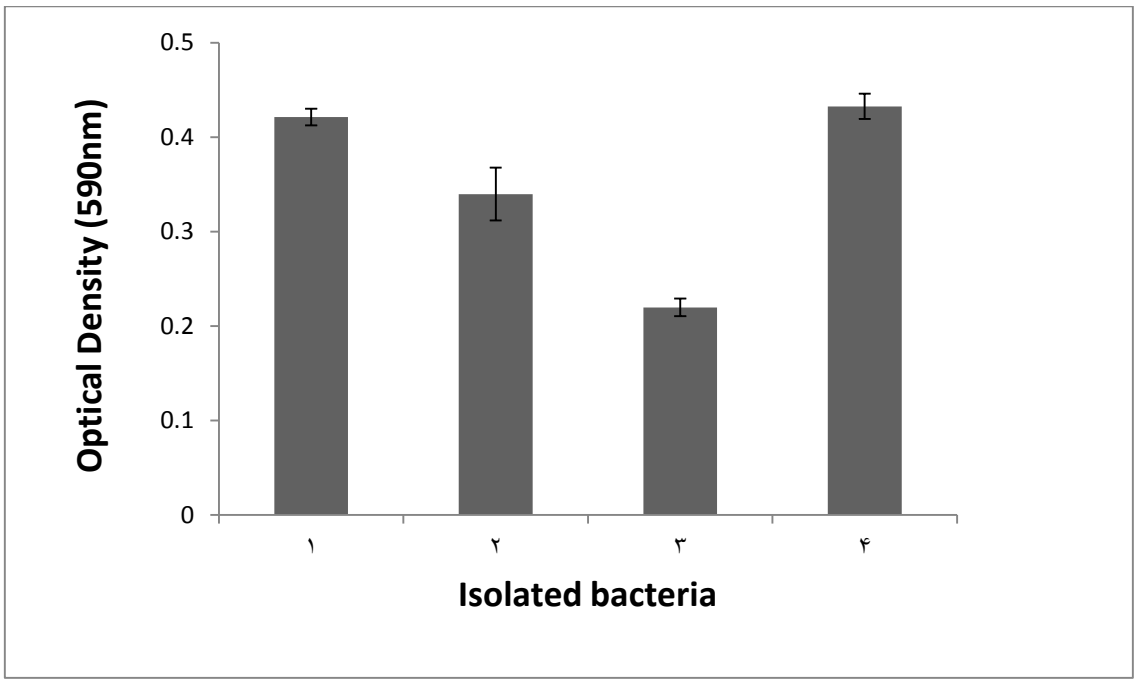

Figure 2: Quantification of biofilm produced by isolated bacteria. The optical densities were measured after solubilization of crystal violet, with the abundance of biofilm considered directly proportional to the optical density reading. All measurements were made in triplicate and the average was calculated for each strain. Error bars represent the standard deviations. Bacterial isolates: 1, *Vibrio* sp. Persian 1; 2, *Vibrio* sp. Persian 2; 3, *Vibrio* sp. Persian 3; 4, *Vibrio* sp. Persian 4.

Discussion

Bioluminescent bacteria are found in nature, their habitat ranging from marine (*V.fischeri*) to terrestrial (*Photorhabdus luminescens*) environments (D'Souza, 2001). Bioluminescence functions are quite clear for higher organisms, whereas the ecological significance of bacterial luminescence is less straightforward. Luminous bacteria distributed in the marine environment have been isolated from various sources including seawater, the light organs and various other parts of marine luminous organisms (Shimomura, 2006). Pujalte *et al*. (1999) suggested that the association with guest organisms might contribute to survival and distribution of luminous bacteria in the marine environment. Studies on luminous bacteria as saprophytic (Davis and Sizemore, 1982), symbiotic (Boettcher and Ruby, 1990; McCann *et al*., 2003) and parasitic (Oliver *et al*., 1986) forms have long been reported in several investigations. However, in the course of our study we focused on isolating free living bioluminescent bacteria. Such luminous bacteria can be isolated readily from marine environments and the present investigation highlights the occurrence of bioluminescent bacteria in an indigenous marine environment based on the observation of luminous colonies in a dark room. Our study represents a first attempt at isolating luminescent bacteria from Sea of Oman, south of Iran. Since in shallow sunlit regions of the oceans, biomass and organic compounds are required to support large accumulations of autotrophic, mixotrophic, and heterotrophic organisms (Haddock *et al*., 2010) and bioluminescent bacteria are

common in the ocean, especially in temperate to warmer waters (Dunlap and Kita-Tsukamoto, 2006), coastal region of Chah Bahar Port, with warm and humid summers and moderate winters, was chosen for water sampling location. Since most species of marine luminous bacteria can be found in more than one habitat (Herring, 2002), luminous bacteria, isolated from layers of sea water as free-living bacteria, might have established one of the possible relationships with other marine organisms in some stages of their lives. In a similar study by Omeroglu and Karaboz (2012), 20 strains of bioluminecscent *V. gigantis* were collected from different ecological niches: sea water, sediment, and sea fish samples. In the present study luminous bacterial isolates grew abundantly on a SWC medium, in which sea water was used in order to support the salts needed for bacterial growth. In an experiment performed by Budsberg *et al*. (2003) SWC was supplemented with inorganic salts such as NaCl, $MgCl_2$, $MgSO_4$, and KCl for bacterial culture. As illustrated in Table 1, all isolated luminous bacteria had the same features in their biochemical tests, with the exception of gelatinase activity, sucrose fermentation and colony color on TCBS agar, though these characteristics could not definitely and precisely conduct us to exact identification of bacteria. In this study the sequencing of 16S rRNA gene was performed by using universal primers which could amplify about 1400 bps sequence. In a similar study, a luminous bacteria from coast of Karachi was identified as *V. harveyi* strain N6 by using a primer pair which could amplify a 550 bps fragment (Nawaz and Ahmed, 2011). All the bioluminescent bacteria isolated and identified in the present study belonged to genus *Vibrio*, a widespread genus of bacteria in coastal waters which is comprised of more than 63 species (Thompson *et al*., 2004). The characterized bacteria were submitted in GenBank Database as new isolates based on their 16S rRNA gene sequences. As part of the study, two other characteristics of isolated bacteria were determined, amount of light production and biofilm formation. Results revealed that among four isolated *Vibrio* species, *Vibrio* sp. Persian 1showed greatest light emission followed by *Vibrio* sp. Persian 2, *Vibrio* sp. Persian 4 and *Vibrio* sp. Persian 3. These data revealed that there are other factors affecting light emission in luminous bacteria isolated from the same environment, with similar characteristics, and belong to the same genus. In other words, amount of light emission from different isolates belong to same genus, *Vibrio*, is variable, though bioluminecsence is under the control of a fundamental similar genetic system. Biofilm formation, a process regulated by quorum sensing along with bioluminescence, conjugation, virulence, and antibiotic production (Miller and Bassler, 2001), was assayed for all isolated bacterial strains. According to Fig. 2, three of the strains examined, isolated from both subsurface and bottom layers, *Vibrio* sp. Persian 1, *Vibrio* sp. Persian 2, and *Vibrio* sp. Persian 4, were moderate biofilm producer, while *Vibrio* sp Persian 3, isolated from bottom layer, was a weak biofilm producer. These data suggested that biofilm formation ability was not affected by the depth of water sampling.

Furthermore, according to previous findings, several Vibrio species such as *V. harveyi*, which can exist free swimming in seawater, adhered to abiotic surfaces, as a constituent of biofilm consortia in marine animals, and in pathogenic associations with marine hosts (Nealson and Hastings, 1979, Mirbakhsh, *et al*., 2014), and *V. fischeri*, which exists in a free-living stage (seawater) or as a mutualist of sepiolid squids and monocentrid fishes (Ruby and McFall-Ngai, 1999; Nishiguchi *et al*., 2004; Nyholm and Nishiguchi 2008), can form biofilm as a ubiquitous phenomenon in aquatic ecosystems. Our results indicated that not only bacteria in association with biotic or abiotic surfaces are capable of forming biofilm but also free living bacteria have the potential to produce biofilm. Other studies which focused on different aspects of biofilm formation by both free-living and symbiont *Vibrio* species represented such capability (Nair and Nishiguchi, 2009; Chavez-Dozal *et al*., 2013). Furthermore, the weakest biofilm producer, *Vibrio* sp. Persian 3, had the weakest potential to emit light, though such correlation was not observed for the greatest light emitter, *Vibrio* sp. Persian 1. The present study showed that screening of south marine environment of Iran for luminous bacteria for the first time, led to the isolation and identification of new bioluminescent bacteria, and revealed Chah Bahar Port, north-east of Oman Sea, as an important ecosystem for these bacteria. More experiments on molecular and biochemical aspects of the isolated bacteria will reflect their capacity, application and functional properties.

In conclusion, the isolation of luminescent bacteria from Chah Bahar Port, as an indigenous discovery, along with increasing rate of exploring new findings about possible applications of bioluminescence in many fundamental biological sciences hold a great promise for improvement of indigenous biotechnological tools. On the other hand, finding novel strains with desirable functional characteristics and biotechnological applications in unscreened marine environment is in progress and may conduct us to explore the new aspects of bioluminescence in the future.

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