In silico Study of Putative Iron Acquisition Systems and Putative Hemolysin Genes of *Vibrio alginolyticus* ATCC17749.

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ABSTRACT

Iron is a most essential micro-nutrient element that is required by microorganisms including *Vibrio* species. The mechanism of iron acquisition is complex by its existence in nature, insoluble ferric complexes, and its high binding affinity by some proteins, specialized for iron-binding protein in the host. *Vibrio* species have evolved various iron transport systems, that permit bacteria to uptake iron from the growing medium. The present study is aimed at the identification of putative iron transport systems, iron source utilization, and putative hemolysin gene. The annotations of the whole genome of *V. alginolyticus* ATCC 17749 analyzed revealed two putative iron transport: iron-siderophore transport system and heme and hemin transport system. Homologs of FhuABCD iron transport system and heme uptake locus *hutZ* and *hutX* were found in the genome of *V. alginolyticus* ATCC 17749. It utilized heme, hemin ferrous sulfate, and ferrioxamine in the iron-depleted growing environment. It's containing five putative hemolysins which are 99% identical to query hemolysins that are found in *Vibrio* species. Four putative hemolysins were present in one chromosome and one in another, these are translated into different functional proteins and may be taking part in the lysis of erythrocytes. All data put together and analyzed recommended that *V. alginolyticus* ATCC 17749 utilized ferrisiderophore and hemin derivative iron sources in an iron-depleted environment. These studies need to be further confirmed mutant library construction and screening of several gene specific mutants.

Keywords: Iron utilization, Heme, Hemin, Iron transport, Hemolysin

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1. INTRODUCTION

Vibrio alginolyticus is a Gram-negative halophilic bacterium, found in marine environments like estuaries and coastal areas. It's been characterized as a frequent pathogen isolated from ill fish with bacterial septicemia-like symptoms. Ulcer illness in marine animals, especially fish, is caused by *V. alginolyticus.* (1). In 1973, it was isolated from the patient with gastroenteritis, wound of the calf, and purulent discharge of conjunctivae, designated as an opportunistic pathogen for human (2). *V. alginolyticus* is presently ubiquitous in seawater and cause wound and ear infection, the infection rate of *V. alginolyticus* in summer season is more prone than winter season (3). The mechanism of infection and transmission of *V. alginolyticus* remains unclear but an indication of the transmission pathway goes through the seawater (4). Scientific communities from China, Europe, and America have also highlighted the virulence of *V. alginolyticus* (5). Some studies have shown infection with *V. alginolyticus* has also caused mortality in immunocompromised patients (6).

Iron is the most important micronutrient metal, catalyzes a broad spectrum of biochemical reactions, essential for all microorganisms except *Borrelia burgdorferi* (7). It plays an indispensable role in DNA synthesis along with in electron transport chain. Biological donor ligands such as nitrogen, sulfur, and oxygen easily form complexes with iron. Iron's ability to bind quickly aids its insertion into the active sites of a variety of metabolic proteins (8). In a reducing and oxidizing environment, iron exists in two oxidation states: ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}) (9). The ability of pathogenic bacteria to use iron is precarious for both survival and infection establishment in the host. The bioavailability of iron in an aerobic physiological pH ranges from 10^{-18} M to 10^{-7} M, which is quite below the minimal availability required for bacterial growth. (10). Many bacteria evolved and can transport iron through secreting light molecular weight iron capturing compound popularly known as siderophore or directly uptake heme or soluble ferrous iron (11). Iron plays a crucial role as a factor in bacterial pathogenesis (12). Maximum iron is present as ferric chloride, which is insoluble in water and physiologically inaccessible to bacteria and is present in sufficient concentrations in the environment for bacterial survival. Iron is bound by high-affinity iron-binding proteins, such as transferrin and lactoferrin, in the host, including humans. (13). The availability of free iron in human and other animals' body fluids at physiological pH is very low for survival of bacterial growth (14). Bacteria produce high-affinity iron-binding proteins, designated as siderophores, to scavenge iron from the environment when the iron is scarce. The iron siderophore complex, also known as ferrisiderophore, is a ferrisiderophore that is delivered to bacteria by a specialized iron

transport pathway. The iron or siderophore acquisition system consists of three transport proteins and one energy provider protein outer membrane protein, a periplasmic binding protein, inner membrane permease, and ATPase proteins respectively (14).

In the present study, we aimed to investigate the putative iron transport and hemolysin gene of *V. alginolyticus* ATCC 17749 through various genotypic and phenotypic analyses *in silico*. We report here the list of putative iron transport genes, hemolysin genes, and iron source utilization.

2. MATERIALS AND METHODS

2.1 Bacterial strains and media

The strain ATCC 17749 of *Vibrio alginolyticus* was obtained from the American Type Culture Collection (ATCC). Luria Bertani (LB) Broth or LB agar plates supplemented with 1.5 % additional Sodium chloride were used to grow *V. alginolyticus* at 37°C. In their respective growing medium with 25% glycerol, all strains were kept at -80°C.

2.2 Sequence availability

FhuD protein sequence with accession number AAC73263.1, retrieved from NCBI at http://blast.ncbi.nlm.nih.gov/protein was saved in FASTA format for analysis.

2.3 Homology search

FhuD sequences are used as a query sequence for BLAST at http://blast.ncbi.nlm.nih.gov/Blast.cgi compared to nonredundant sequence protein databases. At the above-mentioned address, possible potential conserved domains of FhuD were also explored.

2.4 Sequence alignment and *in silico* analysis

Four *V. alginolyticus* protein sequences with E value = 0 and identity > 60% obtained from a BLAST search were aligned for accurate homology analysis. To evaluate structurally related sequences, the amino acid sequences of FhuD were aligned against the template sequence from the previous search. The complete genome of *V. alginolyticus* ATCC 17749 was downloaded from the NCBI, Bethesda, MD, USA GeneBank (www.ncbi.nlm.nih.gov/genome/browse/). The iron transport system and gene clusters were identified by manual investigation.

2.5 Bacterial culture under iron restriction condition

The iron-restricted condition was created by adding 400 µM 2'2-Bipyridyl, in this condition availability of iron is restricted to bacterial use. All iron solutions (10 µM) were prepared fresh daily ferrous sulfate heptahydrates, Ferric chloride, and ferric dicitrate prepared in double-distilled water while hemin solution is prepared in dilute sodium hydroxide solution (≈ 10 mM) prepared in double-distilled water. The capability of *V. alginolyticus* to utilize iron compounds was tested by a method demonstrated by Field et al for the effect of siderophore on the growth of *Campylobacter jejuni* (15). Bacterial culture was grown in different concentrations of different iron sources such as ferrous sulfate, ferric dicitrate, ferrioxamine siderophore & hemin after iron limitation by 2'2-Bipyridyl (400 µM) in LB+ 1.5 % (w/v) NaCl broth. The culture tube was incubated at 37°C with shaking (200 RPM) for 6 hrs. & the A_{600} measured during growth. The values presented are the means of three independent experiments plus the standard error. Samples were tested in triplicate.

3. RESULTS

3.1 Putative iron acquisition gene/system in *V. alginolyticus*

In silico analysis of the whole genome of *V. alginolyticus* ATCC 17749 (Table 1), shows the presence of iron acquisitions genes, involved in iron transport. The information generated through the NCBI and manual analysis which included in Table 1. Target encoding siderophore sensor and receptor system, heme, hemin uptake, and utilization system, and transport system are among these systems.

3.2 *E. Coli* FhuABCD homologs in *V. alginolyticus*

The FhuABCD iron transport system is responsible for the transportation of iron ferrichrome complex in most pathogenic *E. coli* and *Vibrio* species. Multiple proteins in Gram-negative bacteria assist this complex across both membranes. i.e. FhuA, FhuB, FhuC, FhuD and TonB. The sequence of these proteins present in *E. coli* was retrieved from NCBI database and BLAST within the genome of *V. alginolyticus* ATCC 17749 separately, those showing 90% coverage were listed. Outer membrane protein FhuA showed similarity with locus tag N646_RS02370, N646_RS03735, N646_RS515695, and N646_RS16840. FhuB cytoplasmic membrane permease shows similarity with locus tags N646_RS02365 and N646_RS15710. FhuC is related to the inner cytoplasmic membrane FhuB, showing similarity with locus tag N646_RS16815. Periplasmic binding protein FhuD shows similarity with locus tags N646_RS02360 and N646_RS15705. TonB provides energy for the process, showing similarity with locus tag N646_RS17800.

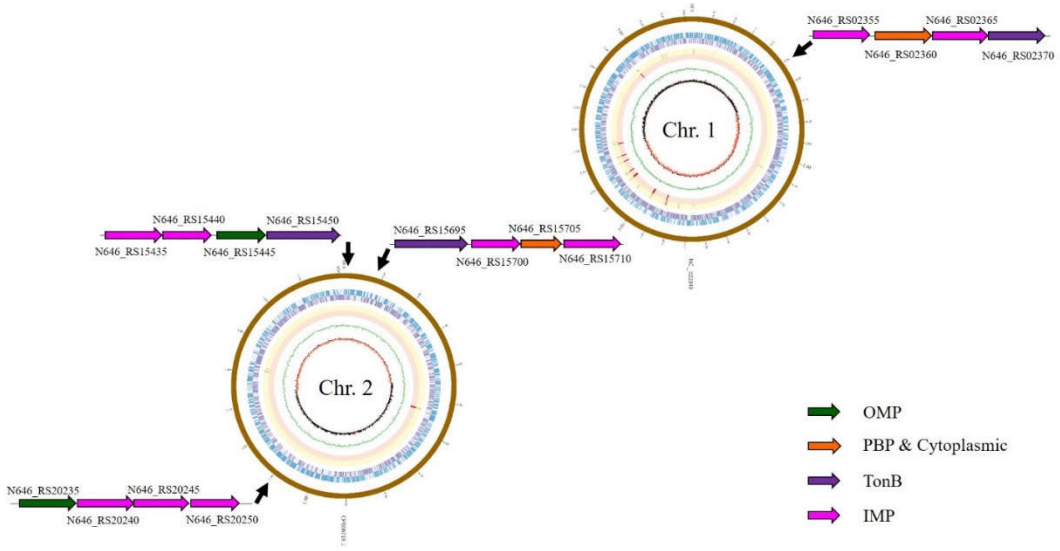

Figure 1. Gene context of *E. Coli* FhuABCD homologs in *V. alginolyticus*

3.3 Iron acquisition genes/systems in *V. alginolyticus*

The location of iron acquisition genes and systems was revealed by *in silico* investigation in *V. alginolyticus* ATCC 17749. The results from gene annotation and analysis of locus tags names are shown in figure 2 and the information on all homolog genes in the genome is mentioned in table 1. *Vibrio species* have multiple iron transport mechanisms that transport the optimal iron from their growing environment. Some of the iron acquisition systems in vibrios are closely linked, indicating that they have a shared evolutionary origin. Other iron transport mechanisms have emerged as a result of natural horizontal gene transfer, and vibrios could have niche-specific iron transport.

Genes of the iron transport system are present on both chromosomes in *V. alginolyticus* ATCC 17749 and encode ferric or ferrous iron transporter, protein for biosynthesis and transport of iron chelator, and unambiguous receptor for various types of chelated iron. The iron transport systems in *V. alginolyticus*, which covered approximately 1 % of the genome, is a quietly similar percentage of *V. cholerae* genome responsible for iron transport systems.

Table 1. Protein involved in iron acquisitions and transport

Chr.	Locus	Locus tag	Protein product	Length	Protein name
1	fhuD	N646_RS02360	WP_005377718.1	294	iron-siderophore ABC transporter substrate-binding protein
1	fhuB	N646_RS02365	WP_017820631.1	658	Fe(3+)-hydroxamate ABC transporter permease FhuB
1		N646_RS02370	WP_017820632.1	740	TonB-dependent siderophore receptor
1		N646_RS03735	WP_017821354.1	711	TonB-dependent siderophore receptor
1		N646_RS03990	WP_017821442.1	359	iron chelate uptake ABC transporter family permease subunit
1	fepG	N646_RS03995	WP_017821441.1	346	iron-enterobactin ABC transporter permease
1	ccmD	N646_RS06345	WP_005380515.1	68	MULTISPECIES: heme exporter protein CcmD
1		N646_RS06350	WP_005380513.1	247	MULTISPECIES: heme ABC transporter permease
1	ccmB	N646_RS06355	WP_005380512.1	222	MULTISPECIES: heme exporter protein CcmB
1	ccmA	N646_RS06360	WP_005380511.1	205	MULTISPECIES: cytochrome c biogenesis heme-transporting ATPase CcmA
1		N646_RS07635	WP_017820405.1	343	ABC transporter ATP-binding protein
1		N646_RS07640	WP_017820404.1	541	iron ABC transporter permease
1		N646_RS07645	WP_005386566.1	337	MULTISPECIES: Fe(3+) ABC transporter substrate-binding protein
1	fur	N646_RS14850	WP_005382141.1	149	MULTISPECIES: ferric iron uptake transcriptional regulator
1		N646_RS14960	WP_005382190.1	75	MULTISPECIES: ferrous iron transport protein A
1	feoB	N646_RS14965	WP_017819946.1	758	Fe(2+) transporter permease subunit FeoB
1		N646_RS14970	WP_005382192.1	76	MULTISPECIES: iron transporter FeoC
2	hutZ	N646_RS15325	WP_005375331.1	176	MULTISPECIES: heme utilization protein HutZ
2	hutX	N646_RS15330	WP_005375329.1	167	MULTISPECIES: heme utilization cystosolic carrier protein HutX
2		N646_RS15335	WP_017821234.1	456	heme anaerobic degradation radical SAM methyltransferase ChuW/HutW
2		N646_RS15340	WP_021707650.1	247	energy transducer TonB
2		N646_RS15345	WP_017821236.1	232	MULTISPECIES: MotA/TolQ/ExbB proton channel family protein
2		N646_RS15350	WP_005375322.1	137	MULTISPECIES: biopolymer transporter ExbD
2		N646_RS15355	WP_005375320.1	289	MULTISPECIES: ABC transporter substrate-binding protein

2		N646_RS15360	WP_005375318.1	345	MULTISPECIES: iron ABC transporter permease
2		N646_RS15365	WP_005385036.1	260	MULTISPECIES: heme ABC transporter ATP-binding protein
2		N646_RS15435	WP_017821244.1	314	iron chelate uptake ABC transporter family permease subunit
2		N646_RS15440	WP_017821245.1	317	iron chelate uptake ABC transporter family permease subunit
2		N646_RS15445	WP_017821246.1	322	siderophore ABC transporter substrate-binding protein
2		N646_RS15450	WP_005375288.1	726	TonB-dependent receptor
2		N646_RS15695	WP_017821853.1	700	TonB-dependent siderophore receptor
2	fhuD	N646_RS15705	WP_017635312.1	309	MULTISPECIES: iron-siderophore ABC transporter substrate-binding protein
2	fhuB	N646_RS15710	WP_017821855.1	655	Fe(3+)-hydroxamate ABC transporter permease FhuB
2	fecE	N646_RS16815	WP_017820095.1	254	Fe(3+) dicitrate ABC transporter ATP-binding protein FecE
2		N646_RS16820	WP_017820094.1	323	iron chelate uptake ABC transporter family permease subunit
2		N646_RS16825	WP_017820093.1	345	iron chelate uptake ABC transporter family permease subunit
2		N646_RS16830	WP_017820092.1	290	Fe(3+)-dicitrate ABC transporter substrate-binding protein FecB
2		N646_RS16835	WP_017820091.1	712	TonB-dependent receptor
2		N646_RS16840	WP_005383467.1	678	TonB-dependent siderophore receptor
2		N646_RS16845	WP_017820090.1	403	hypothetical protein
2		N646_RS17505	WP_017821025.1	257	siderophore-interacting protein
2		N646_RS17800	WP_017819705.1	698	TonB-dependent siderophore receptor TON B
2		N646_RS20235	WP_005376635.1	302	MULTISPECIES: siderophore ABC transporter substrate-binding protein
2		N646_RS20240	WP_017820306.1	311	MULTISPECIES: iron chelate uptake ABC transporter family permease subunit
2		N646_RS20245	WP_005376631.1	316	MULTISPECIES: iron chelate uptake ABC transporter family permease subunit
2		N646_RS20265	WP_017820303.1	668	TonB-dependent siderophore receptor
2		N646_RS21475	WP_017820184.1	723	TonB-dependent receptor
2		N646_RS21480	WP_017820185.1	245	siderophore ferric iron reductase
2		N646_RS22945	WP_017821615.1	128	heme-binding protein

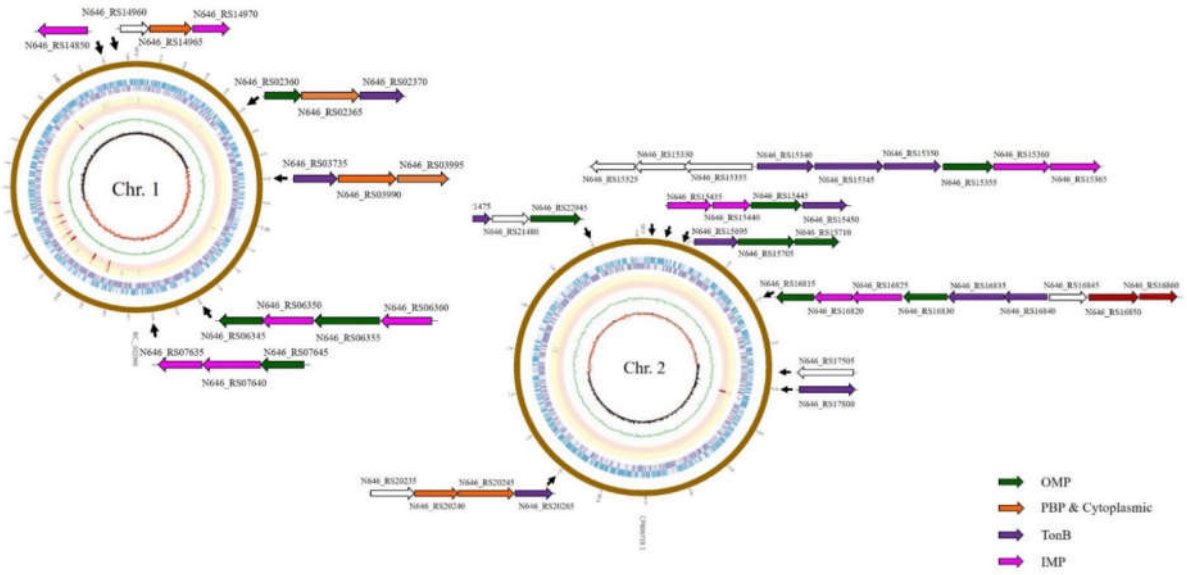

Figure 2. Location of putative iron transport genes in the genome of *V. alginolyticus*

3.4 Putative hemolysins genes of *Vibrio alginolyticus*

Retrieved sequences of hemolysin from NCBI database, which are present in *Vibrio* species. Sequences of hemolysins BLAST within the genome of *Vibrio alginolyticus* ATCC 17749 and selected topmost hit found. Theoretically, those sequences translated into a functional protein means it may be taking a part in hemolysin. We have selected the top 5 hits, all are with 100 % coverage and above 99 % identity. Four putative hemolysin genes are present in chromosome number 1 and one putative hemolysin gene is present in chromosome number 2 (Figure 3).

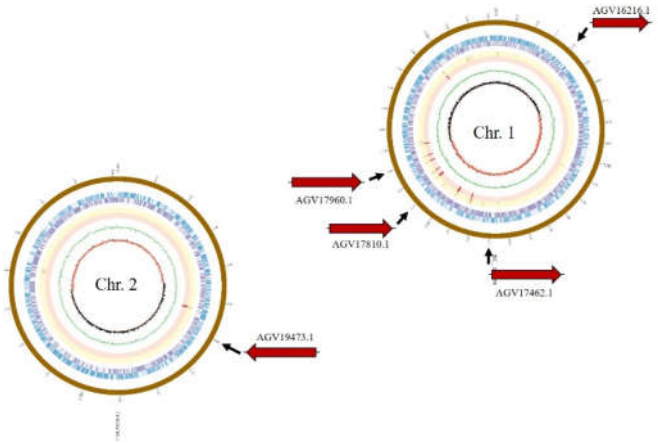

Figure 3. Location of homologs gene of putative hemolysin in *V. alginolyticus* genome

Table 2. Five best homologs of hemolysins in the genome of *Vibrio alginolyticus* ATCC 17749

V. alginolyticus Gene Ref. ID	Coverage %	Identity %	Best BLAST hits	AAs	MW	PI
CP006719.1 **631823-633073** **AGV19473.1**	100	99.76	SGNH/GDSL *Vibrio neocaledonicus* WP_137282845.1	417	47 kD	5.14
CP006718.1 **1745357-1746637** **AGV17462.1**	100	99.77	CBS *Vibrio spp.* JMC 18904 GAJ70819.1	426	47 kD	5.73
CP006718.1 **2326172-2326825** **AGV17960.1**	100	99.08	HlyIII *Vibrio neocaledonicus* WP_137282461.1	217	24 kD	9.24
CP006718.1 **422386-423456** **AGV16216.1**	100	99.72	Hemolysin *Vibrio diabolicus* WP_005393465.1	357	40 kD	4.86
CP006718.1 **2155317-2156450** **AGV17810.1**	100	99.73	HlyC/CorC *Vibrio spp.* WP_005382522.1	377	42 kD	4.91 4.95

3.5 Iron source utilization during iron restriction

The iron utilization assay was also done in a liquid medium in the presence of various iron sources. First, we determine the minimum concentration of 2,2'-Bipyridyl that restrict the growth of *V. alginolyticus* ATCC 17749. Absorbance at 600 nm was used to measure the growth of *V. alginolyticus* ATCC 17749 per hour, and 400 M of 2,2'-Bipyridyl was used to restrict the growth.. The ability of *V. alginolyticus* ATCC 17749 to be stimulated by various iron-containing compounds at the 10 µM concentration was further confirmed by growing in the liquid media. A single colony of *V. alginolyticus* ATCC 17749 from an LB agar plate was inoculated into LB broth then 1% of this culture was inoculated into the growth curve tube containing specific iron-containing compounds. *V. alginolyticus* ATCC 17749 showed a significant level of growth induction by ferrous sulfate, ferrioxamine, hemin, and heme whereas catechol and ferric dicitrate have shown little or no growth stimulation (Figure 4).

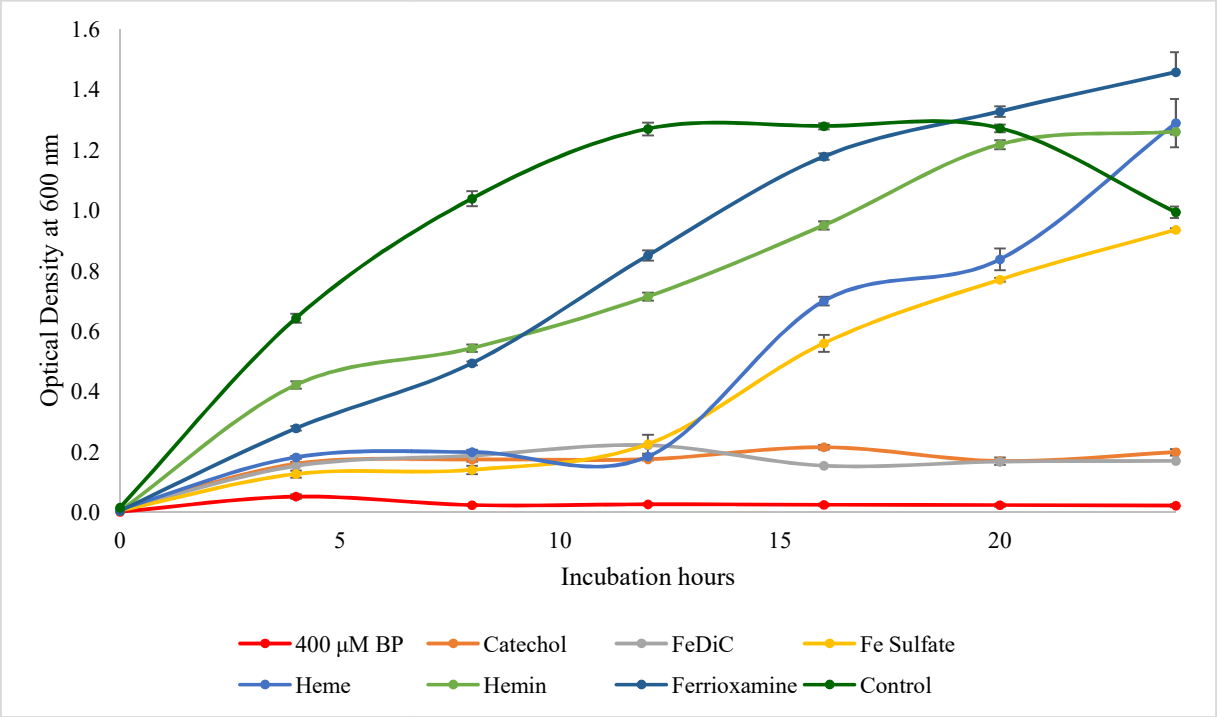

Figure 4. Iron source utilization of *V. alginolyticus* ATCC 17749 in Luria-Bertani broth + 1.5 % NaCl, that was supplemented with 400 µM 2'2-Bipyridyl (Red), catechol (orange), ferric dicitrate (gray), ferrous sulfate (yellow), heme (light blue), hemin (light green), ferrioxamine (dark blue) and control culture (dark green) not supplemented either ferrioxamine or 2'2-Bipyridyl. Optical densities at 600 nm were determined at 4 hours interval during incubation at 37 ºC, 200 rpm and the data plotted are the means of three independent experiments together with the standard error.

4. DISCUSSION

Iron is a most essential micronutrient for the survival of microorganisms with the exception of *Borrelia burgdorferi* (7). Similarly, *Vibrio alginolyticus* also require iron for its growth and survival, it is also an emerging human pathogenic bacteria (16). Every bacteria has one or multiple iron transport systems for iron acquisition from the growing environment (17). Iron transport system FhuABCD present in *E. coli* and *Vibrio* spp, liable for ferrichrome transport (18). Each component or protein of the FhuABCD system FhuA, FhuB, FhuC, FhuD, and TonB has similarities (Table 1) with protein transcribed by the genes/locus tag present in the genome of *V. alginolyticus* ATCC17749. FhuA protein is present in the outer membrane to identify the iron siderophore complex, after the internalization the complex bind to the periplasmic binding protein FhuD, associated with FhuC, and then transported to the cytoplasmic membrane protein FhuB (19). The whole process

requires energy, provided by the TonB, ExbB, and ExbD present in (20). In *E. coli* K-12, the iron transport system FhuABCD has received more attention; it is encoded by a single operon system that includes the genes *fhuA, fhuB, fhuC*, and *fhuD*. (21).

The *in silico* method using gene annotation from NCBI revealed two major iron acquisitions in *V. alginolyticus* ATCC 17749, which recommended that *V. alginolyticus* ATCC 17749 may uptake iron through siderophore transport system and heme-mediated iron transport systems. Besides this locus tags N646_RS16815 and N646_RS14850 produced a protein that is linked to the ferric and Fe3+ dicitrate iron transport system.

In this study, iron depletion was achieved by adding 2,2'-dipyridyl as an iron-chelating chemical compound (22). It forms a complex with iron, causes iron depletion, and depresses the iron-regulated proteins and siderophores. Payne suggested that 100 to 400 µM concentration of 2,2'-dipyridyl may be added to the medium for iron depletion (23), during optimization minimum concentration of 2,2'-dipyridyl that inhibited the *V. alginolyticus* was 400 µM. The iron sources utilized by *V. alginolyticus* ATCC 17749 were identified by the liquid iron utilization assay. Heme, Hemin, ferrous sulfate, and ferrioxamine stimulated the growth of *V. alginolyticus* ATCC 17749 under iron-restricted conditions, with ferric dicitrate and catechol having less effect in inducing the growth of *V. alginolyticus* ATCC 17749. Ferrioxamine and hemin induced sufficient growth under iron-depleted conditions, even more than after twenty hours. Heme and ferrous sulfate-induced sufficient growth but the lag phase were longer than ferrioxamine and hemin. *V. alginolyticus* ATCC 17749 utilize iron sources such as ferrous iron or another iron-holding compound ferrioxamine, hemin, and heme for growth and proliferation. The utilization of heme and hemin by *V. alginolyticus* ATCC 17749 may be transported by the heme uptake gene *hutZ* and *hutX* found in *in silico* analysis. The HutZ is a cytoplasmic protein responsible for heme degradation which is essential for the utilization of heme iron in *Vibrio cholerae* (24). HupA, HvtA, and HupO are heme receptors present in *V. fluvialis* and MhuA in *V. mimicus*, these genes are present in an operon system for HutA and HutR and are same and closely related to *V. cholerae* operon system (25). Utilization of hemin and heme recommended that *V. alginolyticus* ATCC 17749 potentially utilize other iron sources instead of ferrous iron and its infection in the bloodstream, bacterial multiplication within the bloodstream is possible. 200 mg of iron is found in a unit of packed erythrocytes, which fulfill the alternate requirement of iron (26).

In pathogenic vibrios, hemolysins are widely distributed in different types and play various roles in pathogenesis (27). Iron is essential for all microbial growth and replication except *Borrelia burgdoferi*, which also plays an important role in pathogenesis (28). Hemolysins break erythrocytes, which release iron-binding proteins from iron such as hemoglobin, lactoferrin, and transferrin. The iron is captured by the bacteria with the help of a high-affinity iron-binding compound capable of competing with host proteins. The main iron capturing protein is siderophore, a low molecular weight iron-chelating compound, that mainly binds to Fe^{3+} outside of the cell, and

uptake through the outer membrane protein (OMP) into the bacterial cytoplasm (28). The expression of hemolysin protein in marine *vibrio* is controlled in iron limiting conditions, which arise in the host cell during pathogenesis (29). Hemolysin is not limited to only erythrocytes, it also lyses a wide range of immune cells such as mast cells, neutrophils, and polymorphonuclear cells as well as tissue damage to increase virulence (27). Different types of hemolysins produced by the *Vibrio* spp. are not identical but they may be similar. There are four representative families of vibrio hemolysin, including thermostable direct hemolysin (TDH), El or Tor hemolysin (HlyA), thermolabile hemolysin (TLH), and another thermostable hemolysin δ-VPH (30). Sequences from prominent hemolysins from vibrio species were BLAST within the *V. alginolyticus* genome, five locus tags were found those are 99% identical with query sequences. Four locus tag were present in a chromosome and one in another (table 2 & figure 3). These locus tag translated into different functional proteins, may be taking part in lysis of erythrocytes. Out of five, three locus tag were selected for cloning and expression in *E. coli* for further confirmation of hemolysin gene through wet lab experiments.

5. CONCLUSION

It is revealed that *V. alginolyticus* ATCC 17749 expresses mainly two putative iron transport systems, ferrisiderophore and heme, hemin uptake, and utilization system during iron depletion. *In silico* analysis revealed that FhuABCD and TonB are involved in siderophore-based iron transport, while the hutZ, hutX, and TonB systems are involved in heme and hemin transport. Five putative hemolysins are 99 % identical to *Vibrio species* hemolysins and present in both chromosomes. This study is an initial footstep in the direction of understanding to iron acquisition system and hemolysin of *V. alginolyticus* ATCC 17749.

The drawback of the study is that function of every gene or protein involved in the iron transport system in iron repleted and iron-depleted could not be established. However, in future mutant library construction and selection of each gene mutant will be helpful in the understanding of the function of genes. Further investigation of the heme and hemin uptake and utilization system would reveal more evidence on the transportation mechanism of heme and hemin iron sources.

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