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# クルマエビ類で見つかった病原微生物Vibrio nigripulchritudoの性状解析およびゲノム解析


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**Doctoral Dissertation**

# IDENTIFICATION AND GENOMIC CHARACTERIZATION OF A BACTERIAL PATHOGEN *Vibrio nigripulchritudo* ISOLATED FROM PENAEID SHRIMPS

**September 2022**

**Graduate School of Marine Science and Technology**

**Tokyo University of Marine Science and Technology**

**Doctoral Course of Applied Marine Biosciences**

**JAYASUNDARA MUDIYANSELAGE SAJANI NISANSALA RATHNAPALA**

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## Declaration

I hereby declare that this thesis has been composed by myself and is a result of my own investigation. It has neither been accepted, nor submitted for any other degree. All sources of information have been duly acknowledged.

Jayasundara Mudiyanselage Sajani Nisansala Rathnapala

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**Abstract**

The occurrence of bacterial and viral diseases has a severe environmental and economic significance in the shrimp farming industry. Therefore, to control and mitigate the impact of shrimp diseases, viral and bacterial agents should be mainly concerned. Vibriosis is one of the major bacterial diseases that occurred in penaeid shrimps creating spectacular economic losses in the industry. Vibriosis is caused by several *Vibrio* sp. which are categorized as opportunistic pathogens in shrimp farming environments.

*Vibrio nigripulchritudo* is a halophilic, gram negative, oxidative positive bacterium which was first isolated in New Caledonia in 1995 from diseased blue shrimp *Penaeus stylirostris*. First reported event of mass mortality of Kuruma shrimp *P. japonicus* due to *V. nigripulchritudo* infection in Japan was occurred in 2005. In the present study, we isolated three strains of *V. nigripulchritudo* (TUMSAT-V. nig1, TUMSAT-V. nig2, and TUMSAT-V. nig3) from a mass mortality event of white leg shrimps *P. vannamei* occurred in a closed marine aquarium in the Tokyo University of Marine Science and Technology, Tokyo, Japan. For the conventional therapy of vibriosis disease and for proper epidemiological characterization, rapid, sensitive, and specific identification and characterization of the causative organism is required.

For the identification of the causative agents of the bacteria, molecular diagnostic methods were performed including PCR amplification with 16S rRNA and hemolysin genes along with the Sanger sequencing. For the rapid identification, the polyclonal antibodies made from formalin killed cells of *V. nigripulchritudo* were used with agglutination test in this study. Simultaneously, standard challenge tests were conducted to determine the pathogenicity of the isolated bacterial strains for Kuruma shrimp *P. japonicus* through injection and infection challenge models.

After commencing the molecular diagnostics, all the isolated bacterial strains from the diseased shrimps were identified and confirmed as *V. nigripulchritudo* based on the 16S rRNA and hemolysin genes. The obtained sequence data from Sanger sequencing aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn) showed that all the isolated strains of the bacteria (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3) are having the highest homology with the available reference genomes of *V. nigripulchritudo* in gene bank. Concurrently, results of the challenge tests showed that the isolated strains were pathogenically virulent for Kuruma shrimp *P. japonicus* in both immersion and injection infection indicating

that the disease outbreaks due to *V. nigripulchritudo* could be able to cause severe damage to shrimp farming industry.

With the development of technological advances over the last two decades, a remarkable rise of whole genome sequencing technology has contributed to the new generation of sequencing methods. Compared to other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* genome is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. Therefore, Illumina and Nanopore whole genome sequencing technology were used to create hybrid genomic assemblies as the long reads provide information on the genome structure while the short reads provide high base-level accuracy. The genome assembly of the isolated strains of *V. nigripulchritudo* has revealed that all the strains are occupied with two chromosomes (chromosome I and chromosome II) with the presence of either one or two plasmids in their genomes.

Numerous virulence related genes are present in either chromosome I or chromosome II. Adherence is found with Mannose-sensitive hemagglutinin and Type IV pilus while antiphagocytosis is observed with capsular polysaccharides. Flagella is used to maintain chemotaxis and motility in isolated strains. Iron uptake is governed with Vibriobactin, Enterobactin receptors and Heme receptors. For the quorum sensing, auto inducer-1 and cholera auto inducer-2 are found in chromosome I and chromosome II respectively. Bacterial secretion systems are important for secretion of substances as their virulence factors to invade the host cells. The isolated strains are found with EPS type II secretion system and VAS type VI secretion system in their genomes. Hemolysin gene is found in the chromosome II of the isolated strains as a potential virulence factor which causes lysis of red blood cells of the host.

Presence of mobile genetic elements such as prophages, antibiotic resistance genes and genomic islands indicating that these isolated strains of *V. nigripulchritudo* can acquire new genetic information through horizontal gene transfer, which has a significant role in bacterial evolution and affects pathogenesis. The comparative genomic analysis based on whole-genome proteome data of five *V. nigripulchritudo* genomes (three isolated strains with two reference genomes of *V. nigripulchritudo;* TUMSAT-TG-2018 and SFn1-2013) showed that the resulting tree split the *V. nigripulchritudo* strains into three clusters. The higher evolutionary homology is observed in between the TUMSAT-V. nig1 and TUMSAT-TG-2018 followed by TUMSAT-V. nig2 and TUMSAT-V. nig3 respectively while the SFn1-2013 isolated from New Caledonia split into a separate cluster.

With the findings of the genome assembly and annotated genomic features related with the virulence factors and mobile genetic elements of the isolated strains of *V. nigripulchritudo* TUMSAT-V. nig1, TUMSAT-V. nig2, and TUMSAT-V. nig3, we can conclude that the present study regarding the genes and their functions, pathogenicity and host-pathogen interactions and comparative genomics with other related species would allow a complete understanding on pathogenicity and virulence of *V. nigripulchritudo.*

Concurrently, the information on the genetic features, pathogenicity, phylogeny, and evolution of *V. nigripulchritudo* retrieved based on the whole genome sequencing of *V. nigripulchritudo* would be important for the prevention and the control of the spread of the vibriosis disease in penaeid shrimps to secure the seafood production in a sustainable manner.

**Key words:** *V. nigripulchritudo*, whole genome sequencing, virulence, mobile genetic elements, comparative genome analysis

# Chapter 1

## General Introduction

### 1.1 Global aquaculture of Penaeid shrimps

A considerable increase in the present food production is required to overcome the global food insecurity and malnutrition in parallel to the continuous growing of the world population having an expected increase from the current 7.2 billion people to 9.6 billion and 10.9 billion in 2050 and in 2100 respectively (Gerland *et al*., 2014). Fish and other aquatic commodities are considered as vital sources of food for people (Willett *et al*., 2019) as it is one of the cheapest animal protein sources. To achieve the global nutritional goal, world per capita fish consumption was increased from an average of 9.9 kg in the 1960s to 20.5 kg in 2020 with an average annual rate of 2.4 percent (FAO, 2020).

There was a gradual increase in the global fish production over the last couple of decades, where the total fish production in 2020 was 177.8 million metric tons (FAO, 2021). Even though a considerable portion of the total fish production is shared by the capture fisheries, share of capture fishery has stagnated at around 90 million tons since 1990s. However, the demand of fish is increasing in parallel to the population growth, and thus, world aquaculture production has a significant role to fill this gap. Contribution of aquaculture for the world fish production is gradually increasing making almost doubled during the last two decades as its share increased from 25.7 in 2000 to 49.2% in 2020 (FAO,2021).

Different forms of aquatic species, such as fin fish, shellfish, and aquatic plants, are being cultivated in aquaculture systems. Among them, shrimp is one of the popular species cultured in aquaculture sector as it is one of the expensive seafood commodities with a high consumer preference.

With the decline of the naturally available stocks due to over exploitation, there is a growing trend in world shrimp aquaculture production from 4.3 million tons in 2012 to 9.4 million tons in 2018 representing a tremendous growth rate of 7% per year (FAO.2014; FAO, 2020). According to the Global Aquaculture Alliance's GOAL (Global Outlook for Aquaculture Leadership) survey conducted in 2019, shrimp industry showed a recovering growth rate in

2017 and the compound annual growth rate was projected as 5.4% for the period of 2017 to 2021.

The largest shrimp producer is China (37 % out of total world production of shrimps), followed by the Southeast Asian regions (Thailand, Indonesia, and Vietnam with 36 %) and Latin America (Tran *et al*., 2013). Major markets of the internationally traded fishery products are in the United States of America, the European Union and Japan (FAO,2020).

From 1985 to 1995, black tiger shrimp *Penaeus monodon* was considered as the major species of shrimp aquaculture with a contribution of more than 50% to the total cultured shrimp production. Production of *P. monodon* was dramatically declined after 1995 due to the outbreaks of white spot syndrome virus (WSSV) and yellow head virus (YHV) (Le Hong, 2008). Therefore, white leg shrimp *P. vannamei* production was continuously expanded to overcome the production loss. *P. vannamei* was popular among farmers due to their favorable features such as fast growth, low production cost, potentiality for intensive culture and resistance to diseases (Briggs *et al*. 2004, Sellars *et al*., 2015). Beside to *P. vannamei*, Kuruma shrimp *P. japonicus* was widely cultured specifically in the Indo- West Pacific regions (Tsoi *et al*., 2007) although the production is comparatively less than that of other penaeid shrimps (FAO,2018). Among the farmed penaeid shrimp species, the Indian white shrimp *P. indicus* was also cultured and contributed to the global seafood production in a considerable manner (Salam, 2013). However, the global shrimp industry faced an array of challenges over the last couple of decades.

## 1.1 Diseases: key constraint of shrimp farming

Disease outbreaks, feed costs, access to disease-free brood stock, international market prices, climate change and natural disasters are the major challenges of shrimp industry (GOAL 2017). Among these challenges, occurrence of diseases has been identified as the most critical challenge behind shrimp production (Kumar *et al*., 2021) which would directly make significant impact not only to the production itself, but also to the global market. Significant collapses of the industry have been reported in almost all shrimp farming countries such as China, Thailand, Indonesia, Taiwan, and Ecuador (Lucas & Southgate, 2012). Great economic losses were arisen in shrimp farming industry due to the invasion of viruses, bacteria, protozoans, and fungi (Rosenberry, 1998). In accordance with Flegel *et al*. (2008), nearly 60% of production losses were caused by viral agents followed by bacteria (20%). The viral diseases are considered as the most severe infectious diseases of shrimp farming. Massive historical economic losses have

been reported due to outbreaks of monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), yellow head virus (YHV), and Taura syndrome virus (TSV) in Taiwan, Thailand, and America respectively (Liao *et al*., 1992; Brock *et al*., 1997; Lightner, 1996; Flegel, 1997). Concurrently, vibriosis has been identified as one of the most severe bacterial diseases found in aquaculture as the pathogenic or opportunistic *Vibrio* bacteria can create devastating production losses (Goulden *et al*., 2012; Schryver *et al*., 2014; Kumar *et al*., 2020).

Bacterial and viral diseases have a severe environmental and economic significance. Substantial risks are found in amplification of the existing pathogenic strains and spreading of the virulence to commensal bacteria with the intensification of culture systems (Waterfield *et al*., 2004). Therefore, to control and mitigate the impact of the shrimp diseases, viral and bacterial agents should be mainly concerned.

## 1.3 Vibriosis

### 1.3.1 Overview of the disease

Vibriosis also called as *Vibrio* disease, bacterial disease, penaeid bacterial septicaemia, penaeid vibriosis, luminescent vibriosis or red-leg disease (Guzman *et al*., 2004) is considered as one of the major health issues in shrimp farming since early 90's in Indonesia (Sunaryanto and Mariam, 1986), Thailand (Jiravanichpaisal *et al*., 1994), India (Karunasagar *et al*., 1994), Philippines (Lavilla-Pitogo *et al*., 1990), Australia (Pizzutto and Hirst, 1995), Taiwan (Liu *et al.,* 1996), Japan (Lightner *et al*., 1996), and Ecuador (Robertson *et al*., 1998). The causative *Vibrio* species have a wide distribution in culture facilities in both hatcheries and ponds throughout the world (Lightner *et al*., 1992; Lavilla- Pitogo *et al*., 1996; Lavilla-Pitogo *et al*., 1998; Chen *et al*., 2003) creating a global loss of approximately 4.5 billion USD (Chellapandian *et al*., 2021). Although all the life stages of shrimps are susceptible for the disease, early life stages (larvae, post larvae and early juveniles) are particularly prone due to lack of an adaptive immunity system (Gollas – Galvan *et al*., 2017).

Massive outbreak of Vibriosis together with a rapid multiplication of bacteria have been detected in parallel to triggering of environmental factors such as poor water quality, crowding, high water temperature, low dissolved oxygen, and low rate of water exchange. (Wang and Leung, 2000). Vibriosis is highly abundant in warm weather with high salinities and organic

load during the late summer period (Randa *et al.,* 2004). As stated by Balasubramanian *et al.* (2021), probability of the occurrence of viral diseases especially white spot syndrome is also magnified by the presence of high diversity of *Vibrio* species in shrimp ponds causing a severe threat to the seafood industry.

### 1.3.2 Causative agents

Vibriosis is caused by a group of bacteria belongs to phylum Proteobacteria, class Gammaproteobacteria, family Vibrionaceae and genus *Vibrio* (Faruque and Nair, 2006). The "*Vibrio"* is derived from Latin word of "vibrare" having a wave or to vibrate (Farmer III *et al*., 2015). *Vibrio* sp. are Gram-negative, motile, and facultative anaerobe bacteria.

*Vibrio* sp. are frequently abundant in aquatic environments, and they represent approximately 45% of the total bacterial count (Suantika *et al*, 2001). *Vibrio* sp. are found in the normal microbial community of shrimp consisting 85% of bacterial flora in the gut of shrimp (Moss *et al*., 2000). Several *Vibrio* sp. are observed as opportunistic pathogens having a potential to make tremendous economic losses in the shrimp farming industry. Specifically, *Vibrio* sp. have a potential to collapse the entire shrimp production by causing 100% mortality (Sudheesh & Xu, 2001). Various studies have been performed to identify the *Vibrio* species in infected shrimps. According to the previous literature, identified causative agents of the vibriosis of shrimps are *V. harveyi* (Lightner, 1996; Soto-Rodríguez *et al*., 2012), *V. alginolyticus* (Vandenberghe *et al*., 1999), *V. parahaemolyticus* (Tran *et al*., 2013), *V. campbellii* (Soto-Rodríguez *et al*., 2006), *V. nigripulchritudo* (Goarant *et al*., 2006), *V. penaeicida* (Goarant and Merien, 2006), *V. fluvialis, V. damsela* and *V. vulnificus* (Chythanya *et al*., 2002). According to Flegel (2012), lack of best management practices of shrimp farming practices is the major reason behind the serious infections of opportunistic pathogens of *Vibrio* sp. The isolation of *Vibrio* sp. has been performed from different shrimp farming systems while they have been detected in an array of organs of different shrimp species worldwide (Table 1.1). Inclusively, the global shrimp production is adversely affected by the *Vibrio* sp. causing a high risk on global food security (Cu´ellar-Anjel *et al*., 2014).

Table 1.1: Shrimp diseases caused by or associated with *Vibrio* species (Adapted from Valente and Wan,2021).

| Diseases | Common *Vibrio* species | Known susceptible shrimp species | References |
|---|---|---|---|
| Septic Hepatopancreatic Necrosis Disease (Hatchery and grow-out) | *V. alginolyticus*<br>*V. campbellii*<br>*V. harveyi*<br>*V. parahaemolyticus*<br>*V. penaeicida*<br>*V. vulnificus* | All farmed shrimp | Morales-Covarrubias and Gomez-Gil (2014)<br>Stern and Sonnenholzner (2014)<br>Morales-Covarrubias *et al*. (2018) |
| Luminescent vibriosis (eggs and larvae) | *V. campbellii*<br>*V. harveyi*<br>*V. splendidus* | *P. monodon*<br>*L. vannamei*<br>*P.indicus* | Lavilla-Pitogo *et al*. (1990)<br>Diggles *et al*. (2000) |
| Shell disease (Juvenile and adults) | Chitinolytic *Vibrio* spp. including<br>*V. alginolyticus*<br>*V. anguillarum*<br>*V. fluvialis*<br>*V. harveyi-like*<br>*V. mimicus*<br>*V. parahaemolyticus*<br>*V. splendidus*<br>*V. vulnificus* | *Macrobrachium rosenbergii*<br>*Penaeus* sp. | Lavilla-Pitogo and de la Pe˜na (2004)<br>Jayasree *et al*. (2006) |
| Summer syndrome shrimp (Grow-out) | *V. nigripulchritudo* | *P. japonicus*<br>*P. stylirostris*<br>*L. vannamei* | Goarant *et al*. (2006)<br>Sakai *et al*. (2007) |

### 1.3.3 Clinical signs and histopathology

Shrimps infected with vibriosis can be identified by the appearance of cloudiness of abdominal muscles and the presence of brown spots in the lymphoid organ, tail, and gills. Further, dark skin with ulceration, tissue and appendage necrosis, lethargy, slow growth, slow larval metamorphosis and body malformation, bioluminescence, muscle opacity with melanization followed by empty mid gut and anorexia are some of the other clinical sings of the Vibriosis (Karunasagar *et al.*1994; Lightner, 1996; Smith, 2000). During the acute phase and severe epizootic outbreaks of vibriosis, inflammation of the organs (hepatopancreas, lymphoid organ, gills, heart) together with many rod-shaped bacterial cells are characterized with the expansion of chromatophores (Valente and Wan, 2021).

Atrophy of the hepatopancreas (shrunken and discoloration) with multifocal necrosis was observed in white leg shrimp *P. vannamei* after infection of *V. parahaemolyticus* (Khimmakthong and Sukkarun, 2017). Further, a massive sloughing of hepatopancreas tubule epithelial cells, a massive intertubular hemocytic infiltration and aggregation followed by secondary bacterial infections were also detected.

### 1.3.4 Prophylaxis and treatments

As most of the *Vibrio* sp. are opportunistic, they cause infection when the host is in stressed condition. Hence, an integrated health management plan is required to overcome the disease by ensuring the maintenance of bio security in the farm. As stated by Pridgeon and Klesius (2012), integration of good husbandry and farm management practices composed of proper water quality management strategies via water treatment and bio augmentation, provision of good quality feeds, use of pathogen free and disease resistance brooders can be served as good strategies to prevent disease outbreaks in aquaculture production systems. The most cost-effective method to manage vibriosis is the prevention of the disease before it enters the farm (Defoirdt *et al*. 2011).

Heavy efforts must be performed to control the disease outbreaks once it is diagnosed in the farm. Usage of antibiotics are extensively applied in controlling the bacterial diseases in aquaculture. Although antibiotics such as chloramphenicol, cotrimoxazole, streptomycin, and erythromycin were used to combat *V. harveyi* infection in shrimps, the bacterium became resistant making the antibiotic is no longer effective (Defoirdt *et al*.,2011). Application of green water technique is relatively a novel method to control the blooming of *Vibrio* sp. in shrimp

culture ponds (Tendencia and dela Pena, 2003). Also, use of lytic bacteriophage against the *Vibrio* sp. revealed successful decreasing of 90% of *Vibrio* population after 4 hours of treatment (Solıs-Sanchez *et al*.,2016). Similarly, application of probiotics such as *Pseudomonas fluorescens, Bacillus subtilis, Lactobacillus plantarum*, and *Streptomyces* sp. has reduced the mortality caused by vibriosis in fish and shrimps in an effective way (Frans *et al*. 2011; Touraki *et al*. 2012; Tan *et al*. 2016). Use of quorum sensing inhibitors (Defoirdt, 2018), immune priming (Wongtavatchai *et al*., 2010), and use of natural products and bio active compounds (Dang *et al*., 2019) are prophylaxis and therapeutic novel tools applied to combat vibriosis in shrimps.

Among the *Vibrio* sp. causing the vibriosis disease in penaeid shrimps, *V. nigripulchritudo* is considered as an emerging opportunistic pathogen causing high mortalities in penaeid shrimps. Therefore, the present study is specifically designed to study about the genomic characterization and virulence of *V. nigripulchritudo.*

### 1.4 *Vibrio nigripulchritudo*

#### 1.4.1 Taxonomy and classification

*V. nigripulchritudo* also known as *Beneckea nigripulchritudo, B. nigrapulchritoda,* and *B. nigrapulchrituda* is a bacterium classified under the family Vibrionaceae, class Gammaproteobacteria, and phylum Proteobacteria (Baumann *et al*., 1980). The Latin meaning of *nigrapulchritudo* is “black beauty” refereeing to the presence of blue-black colonies on basal medium agar with 0.2% (v/v) glycerol.

Regarding the DNA-DNA hybridization experiments conducted by Reichelt *et al*. (1976), it has been found that the *V. nigripulchritudo* is highly related (91-96%) to the type strains and less than 20% related with the 15 other *Vibrio* sp. As stated by Baumann *et al*. (1980), *V. nigripulchritudo* is included with the marine group of *Vibrio* depending on the 16S rRNA gene sequence analysis and immunological relationship of superoxide dismutase. Also, the closest relative of *V. nigripulchritudo* was identified as *V. penaeicida* with 80% to 90% nucleotide identity relatedness found by DNA hybridization suggesting that these species comprise a distinct group of shrimp-associated bacteria in the genus *Vibrio* (Kawato *et al*., 2018).

### 1.4.3 Isolation of the *V. nigripulchritudo*

The first isolation of *V. nigripulchritudo* was performed in New Caledonia in 1995 from diseased blue shrimp *P. stylirostris* affected by winter vibriosis syndrome 93 (Costa *et al*., 1998) which was found only in two adjoining farms suggesting a geographically restricted phenomenon (Goarant *et al*., 1999). Nevertheless, the second isolation of *V. nigripulchritudo* was brought in 1997 in moribund blue shrimp *P. stylirostris from* another shrimp farm which was located 50 km away from the farm where *V. nigripulchritudo* was originally isolated. At the second time, the disease was not associated with the syndrome 93 epidemiology which usually occur when water temperature is below 25.8 °C. According to the histopathological findings of diseased shrimps, the disease was considered as "Summer Syndrome", an acute systemic vibriosis caused by *V. nigripulchritudo* with a highly stereotyped epidemiology causing high mortalities in cultured shrimps (Goarant *et al*.,2000).

Moreover, a mass mortality of Kuruma shrimp *P. japonicus* was occurred in 2005 in Japan, and the causative agent was identified as *V. nigripulchritudo* which spread during the high-water temperature (around 27 °C). It was the first reported event of *V. nigripulchritudo* isolation from in Kuruma shrimp farms in Japan. As stated by Gorrant *et al*. (2006), Summer Syndrome caused by *V. nigripulchritudo* is occurred with high temperature ranging from 20 -30 °C implicating that the severity of the disease is enhanced when the water temperature is high. Hence, this disease must be seriously addressed in present along with the global warming.

### 1.4.2 Identification of *V. nigripulchritudo*

Rapid, sensitive, and specific identification and characterization of the causative organism is indispensable for the conventional therapy of the vibriosis and for proper epidemiological characterization. *V. nigripulchritudo* can be identified using different identification techniques including presumptive identification with suitable culture medium, biochemical identification, serological identification using monoclonal antibodies and molecular methods.

Despite it is time consuming, biochemical tests are being still considered as a conventional method of *Vibrio* sp. identification with the aid of different biochemical properties (Austin and Austin, 1999). *V. nigripulchritudo* is a halophilic, Gram negative, oxidative positive and O/129 sensitive bacteria which can be grown in a temperature range from 20-30 °C (optimum temperature: 25 °C) and a salinity range of 3-5 ppt (Sakai *et al*., 2007). The bacteria are motile as they consist of a sheathed polar flagellum (Baumann *et al*., 1980). Colonies on brain heart

infusion agar (5% NaCl) appear to be low convex, entire, circular, semi-transparent cream-colored colonies (Costa *et al*., 1998) while producing light greenish color colonies in Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar. As stated by Sakai *et al*. (2007), biological and biochemical characteristics of the isolates obtained from shrimp infected with *V. tapetis* and *V. nigripulchritudo* exhibited similar characteristics as shown in the Table 1.2. *V. tapetis* can be distinguished from *V. nigripulchritudo* as *V. tapetis* prefers 4 °C but not in 30 °C. (Borrego *et al*., 1996).

Table 1.2: Comparison of the biological and biochemical characteristics of the isolates collected from shrimp infected with *V. tapetis* and *V. nigripulchritudo* (adopted from Sakai *et al*., 2007)

| Characteristics | *V. nigripulchritudo**[3] | *V. tapetis**[4] |
|---|---|---|
| Salinity range of growth (%) | 3-5 | 1-5 |
| Temperature range of growth (°C) | 20-30 | 4-22 |
| O/129 test | S | S |
| Oxidase | + | + |
| B- Galactosidase | + | + |
| Arginine dihydrolase | - | - |
| Lysine decarboxylase | - | - |
| Ornithine decarboxylase | - | - |
| Citrate | + | - |
| H2S production | nd*[3] | - |
| Urease | - | - |
| Tryptophan deaminase | Nd | - |
| Indole production | + | + |
| Voges- Proskauer | - | - |
| Gelatinase | + | + |
| D-Glucose | + | + |
| D- Mannitol | + | - |

| Inositol | + | - |
| --- | --- | --- |
| D-Sorbitol | + | - |
| I-Rhamnoso | - | - |
| Sucrose | - | - |
| D- Molibioco | - | - |
| D- Amygdalin | + | + |
| L-Arabinose | - | - |

*[1]S: sensitive
*[2]: symbols +: positive, -: negative
*[3]: Buller, 2004
*[4]: Castro *et al*., 1992, Borrego *et al*., 1996, Buller, 2004
*[5] nd: not determined

Polyclonal antibodies prepared against sodium azide- killed cells can be applied as a rapid and specific method to identify different *Vibrio* sp. having similar phenotypical, and biochemical properties as this method is highly specific for those species. Application of antibodies in the detection of *Vibrio* sp. is popular among scientists as this method is cheap and less time consuming (Wangman *et al*., 2021). Therefore, this method can also be applicable to identify the causative strains of *V. nigripulchritudo.*

The conventional microbiological methods of detection, identification and characterization of *Vibrio* pathogens are time consuming, and the results of those methods are sometimes either with false-negative or false-positive results. To overcome these challenges of the conventional methods, different types of molecular detection methods have been developed over the last decade to get more accurate and faster results (Gdoura *et al*., 2016). Although the phenotypic identification of *V. nigripulchritudo* can be conducted easily, it lacks with the sufficient discriminating power for epidemiological studies (Goarant *et al*., 2006). Thus, for a proper identification and typing of *Vibrio* sp., various types of molecular methods have been employed together with a higher discriminatory power and higher reproducibility (Gomez *et al*., 2009).

To study the bacterial phylogeny and taxonomy, 16S rRNA gene sequence is widely used as a common housekeeping genetic marker. Due to the presence of 16S r-RNA in almost all bacteria, consistent nature of the gene over time, and large fragment size (1500 bp) for informatic

purposes, 16S r-RNA gene sequence is considered as one of the best sequences to identify species and genus of isolates (Janda and Abbott, 2007). For instance, Sakai *et al*. (2007) identified *V. nigripulchritudo* which was isolated from Japan in 2005 by using the 16S rRNA sequence, and it gave the highest identity (99%) with the available *V. nigripulchritudo* ATCC27043 showing identical patterns in DNA hybridization (Gene bank accession number: X74717).

For characterizing the isolates of bacterial and fungal species by relying on the availability of nucleotide sequence data obtained from housekeeping and non-housekeeping genes, the Multi Locus Sequence Typing (MLST) is used (Sullivan *et al*., 2005). As stated by Goarant *et al*. (2006), the MLST and AP-PCR techniques were used to distinguish four clusters of *V. nigripulchritudo* (cluster A, B, C and D) with different pathological features which were isolated from infected shrimp collected in New Caledonia.

As the traditional 16S rRNA sequence is rather laborious and costly, advancements in sequencing technologies are developed with greater numbers of sequencing reads at much lower costs for the characterization of microbial communities (Degnan and Ochman, 2012). A substantial opportunity is provided with whole genome sequencing technique to explore the emergence of pathogens yielding novel insights into pathogenic evolution (Fu *et al*., 2021). Results of the high-throughput sequencing-based comparative genome analysis using Illumina HiSeq showed that isolated strain of *V. nigripulchritudo* (SFn1) has shown that the genome consists with two circular chromosomes of 4.1 Mb and 2.2 Mb with an average percent G+C content of 45.9 % and 45.5 %, respectively and having 02 plasmids (250 kb and 11 kb) with a total of 5,653 predicted coding sequences (CDSs) in both chromosomes and plasmids. Also, the results indicated that in highly pathogenic strains belonging to clade A, both plasmids are present while only one plasmid (260 kb) was observed in moderately pathogenic strains. Finally, all the isolates (Highly pathogenic -HP, moderately pathogenic-MP and non- pathogenic-NP) were categorized into three clades with very little intra-clade diversity. All the HP strains followed by some MP and NP are categorized in clades A and B while the isolate from Madagascar is joined to a different clade named as clade M (Goudene`ge *et al*., 2013).

### 1.4.5 Virulence of *V. nigripulchritudo*

As stated by Goarant *et al*. (2006), MLST revealed that all isolates of monophyletic *V. nigripulchritudo* from shrimp affected by Summer Syndrome were identified as moderately

pathogenic (20–80% mortality; MP) or highly pathogenic (80–100% mortality; HP) and nonpathogenic isolates (NP, 0–20% mortality). Reynaud *et al*. (2008) found that the virulence of *V. nigripulchritudo* towards the blue shrimp *P. stylirostris* has been correlated with the presence of a small plasmid, pB1067. However, a second plasmid, pA1066 present in *V. nigripulchritudo* isolate is also served with high pathogenicity in HP phenotype demonstrating full virulence in several models of infection. Concurrently, it has been found that the strains containing both plasmids (pB1067 and pA1066) induced the highest level of mortality of infected shrimps indicating that the high pathogenicity of *V. nigripulchritudo* for *P. stylirostris* (Le Roux *et al*., 2011). Also, the same researchers have indicated that a putative MARTX toxin and its transporter are encoded in one gene cluster within pA1066 with a contribution to virulence towards the infected shrimps which was associated with the virulence of *V. cholerae* (Lin *et al*., 1999), *V. vulnificus* (Liu *et al*., 2007) and *V. anguillarum* (Li *et al*., 2008)

HP strains are encoded with galactose utilization operon (HP1), a siderophore ABC transporter, a peptidase (HP2) and genes involved in purine metabolism (HP3) and proteins annotated as putative toxins (HP4) while the MP strains are found with MP-specific genes for ABC transporters, metabolic proteins, and several proteins of unknown function. A putative toxin-encoding gene, VIBNI_pA0182 (82.9 kDa) was identified in HP4 module which is correlated to the molecular mass of the toxic proteinaceous compound found in the isolated V. nigripulchritudo strain-SFn1 culture supernatant which creates the virulence. Hence, the toxin was named as nigritoxin which is a key factor of *V. nigripulchritudo* shrimp pathogenesis shared by different lineages (Goudene`ge *et al*., 2013). Therefore, plausible avenue for parallel evolution from harmless to pathogenic bacteria is possible with the acquisition of nigritoxin which is in a large mobile plasmid.

## 1.5 Bacterial genome characterization and annotation

### 1.5.1 Genome sequencing technologies

The complete genetic information of an organism or a cell is called as the genome where the single or double stranded nucleic acids (DNA and RNA) stored the information in a linear or circular sequence. Efficient technologies were developed to increase the accuracy, throughput, and sequencing speed to precisely determine the genome sequence of a particular organism. The first organism with whole genome sequenced was identified as Bacteriophage φX174

(Sanger, 1977). As stated by Fleischmann *et al*. (1995), *Haemophilus influenzae* was reported as the first living organisms which the whole genome was sequenced by Craig Venter in the 1980s.

Numerous possible progresses on DNA sequencing were made with the pioneer works performed by Paul Berg, Frederick Sanger, and Walter Gilbert, with the development of the Sanger's 'chain-termination' sequencing technology, most widely known as Sanger sequencing (Sanger *et al*., 1977). Cheaper and faster sequencing techniques were searched by the researchers as the Sanger sequencing was limited in speed and cost owing to its reliance on dideoxy nucleotide (ddNTP) terminators and sequence of a single DNA fragment at a time (Shendure *et al*.,2005).

With the development of technological advances over the last two decades, a remarkable rising of DNA sequencing technology has contributed to the new generation of sequencing methods which were targeted to complement and replace the Sanger sequencing eventually. The new technology was named as either next-generation sequencing (NGS) or massively parallel sequencing (MPS) with a wide diversity of approaches. These techniques can be used to generate massive amounts of data in a faster and cost-effective way. At present, numerous brands are found as Illumina, Ion Torrent (Thermo Fischer Scientific), BGI Genomics, PacBio and Oxford Nanopore Technologies (Pereira *et al*., 2020) in the global NGS market.

A group of cyclic-array sequencing technologies mainly included with the Illumina and Ion Torrent platforms are belonged to the second-generation platforms composed with the preparation and amplification of libraries (prepared from DNA/RNA samples), clonal expansion, sequencing, and analysis (Shendure and Ji, 2008). To eliminate the limitations of PCR-based methods, such as nucleotide misincorporation by a polymerase, chimera formation and allelic dropouts (preferential amplification of one allele) causing an artificial homozygosity call, the introduction of the third generation NGS technology was brought.

Helicos Genetic Analysis System was the first commercial 3$^{rd}$-generation sequencer to sequence every strand in uniquely and independently (Thompson and Steinmann, 2010). However, due to its slow speed and relatively expensive nature, it does not exist for a long time in the market. PacBio RS II sequencer was introduced in 2011 with the concept of single-molecule real-time (SMRT) sequencing which enables the sequence of long reads with average read lengths up to 30 kb (McCarthy, 2010). In 2015, the Oxford Nanopore Technologies commercialized the second approach to a single-molecule sequencing named MinION which relies on the electrical changes in current as each nucleotide (A, C, T and G) which passes through the nanopore

(Deamer *et al*., 2016). Electrophoresis is used to transport an unknown sample via a small opening with the ionic current passing through it and the change of the current is used for identification of each molecule and to perform the sequencing (Jain *et al*., 2015).

### 1.5.2 Genome diversity

Huge wealth of bacterial genomic data collected from all over the world with a capability of sharing openly is resulted with the rapid development of high-throughput sequencing. Genomic sequence data are included in the DNA Data Bank of Japan (DDBJ), the European Bioinformatics Institute (EMBL-EBI), and the National Centre for Biotechnology Information (NCBI) who are the members of the International Nucleotide Sequence Database Collaboration (INSDC) (Danchin *et al*., 2016). Comparison of the available genomic data showed that there are wide variations in the organization of the genome of a particular organism. Typical belief of the presence of a single circular chromosome with some of circular plasmids in the genome of bacteria must be revised with the availability of genomic sequencing data. For instance, the *V. parahaemolyticus* FORC_014 strain consists with two circular chromosomes (3,241,330 bp for chromosome 1 and 1,997,247 bp for chromosome 2), one plasmid (51,383 bp), and one putative phage sequence (96,896 bp) (Ahn *et al*., 2016) while an average of ~0.9-mb linear chromosome and 19 linear and circular plasmids with a total size greater than 0.56 mb are found in the genome of *Borrelia burgdorferi* (Travisano, 2001).

The variations of the G+C content was also observed in different pathogenic organisms. For instance, the G+C content of *B. burgdorferi* is 29 percent while 65 percent in *Mycobacterium tuberculosis* which indicates the uniqueness of the pathogenicity of a respective strain (Fleischmann *et al*., 2002). Also, usually half of the predicted coding sequences (CDCs) consist of an unknown function while half of the CDCs are unique to a particular organism. This phenomenon also exemplified the diversity of microbial gene which emphasizes our limited knowledge about genes and genomic functions of a microbial pathogen.

The sequencing techniques are used to compare the gene content, genomic organization, and gene expression within species of multiple strains. Therefore, the comparative study of closely related genomes improves our understanding about the evolutionary processes involved in the emergence of new infectious diseases.

### 1.5.3 Genome annotation

After the generation of a huge amount of DNA sequence information by the next generation sequencing, the information stored in each sequence must be extracted to understand the insights of the respective genome of the organism. Annotation is the process of retrieving the information encoded within different sequence patterns to identify the location and function of those genes (Ejigu and Jung, 2020). Therefore, genome annotation means the annotation of single nucleotides on thousands of individual genomes. With the flourishment of the scope of genome annotation, a wide range of information regarding functional elements as noncoding RNAs, promoter and enhancer sequences, DNA methylation sites, and many more other information is available. Although there are hundreds of the eukaryotic genomes and over 100,000 bacterial genomes available in the GenBank at present, many more genomes are yet to be received (Salzberg, 2019).

Quality of the genome assembly is an essential criterion for obtaining a successful annotation. To get a high-quality genome assembly, the improvements in sequencing, including long-read and linked-read technology have performed (English *et al*., 2012). Use of high-quality genome assemblies has governed the improvements of whole-genome alignments and annotations (Armstrong *et al*., 2019). Automatic annotation systems are important for the annotation of large amounts of sequence data generated by NGS.

#### 1.5.3.1 Structural annotation

Structural annotation means the finding of functional features of DNA such as exons, introns, promoters, transposons etc. As structural annotation is used to find genes in the genomic sequences, the genes related to protein coding, non-coding genes, pseudogenes are identified to improve the understanding of the entire genomes (Zhang *et al*., 2016).

In prokaryote genome, repeats account for 0 percent to over 42 percent which are located adjacent to one other in the centromeres (Barra *et al*., 2018). Identification of the essential features of repetitive elements is still challenging. Availability of repeat masking tools such as RepeatMasker is implemented to mask the repetitive regions before the gene identification (Smit *et al*., 1998). After masking, to align transcript and protein evidence, BLAST is used as the second step of structural annotation before identifying the respective gene (Altschul *et al*., 1990).

Identification of protein-coding genes and other regulatory elements is highly a paramount feature in genome annotation. Gene prediction programs can be categorized into three: ab initio methods (based on nucleotide sequence), homology-based methods (aligns the sequence with expressed sequence tags), and combined methods with the availability of numerous tools (Ejigu and Jung, 2020).

To present the information as evidence of predicted tasks, supporting data are required. With the availability of huge amount of cDNA sequences retrieved from biological databases, homology-based methods play a key role in genome annotation (Sagot *et al*., 2002). The comprehensive public-domain databases; the GenBank, European Nucleotide Archive (ENA) and DNA Data Bank of Japan (DDBJ) readily provide high-quality and freely accessible nucleotide sequences with the functional information (Kodama *et al*., 2018). Also, for protein annotation data, UniProtKB/Swiss-Prot, UniProtKB/TrEMBL, and InterPro provide information on protein families and domains (Mitchell *et al*., 2019).

#### 1.5.3.2 Functional Annotation

Functional annotation is composed with the association of biological information with protein coding gene or protein sequences identified by structural annotation. After identifying a similar sequence using BLAST, association of a functional description with a gene is involved with the functional annotation to assess the variation in gene (Fitihamlak and Jung, 2020). Genomic data generated by the NGS technology are directly annotated by with the automated functional annotation methods using different available data bases.

As stated by Botstein *et al*. (2015), Gene Ontology (GO) is one of the most comprehensive and widely used knowledgebase for gene function which covers three aspects of gene function; (i) molecular function (activity of a gene product at the molecular level), (ii) cellular component (location of the gene product), and (iii) biological process (a biological program, in which a gene's function is used). Concurrently, a link between genomic data and higher-order functional information is created by Kyoto Encyclopedia of Gene and Genomes (KEGG) by affording the knowledge on high-level functions and utilities of the biological system (Kanehisa and Goto, 2000). Also, some other data bases, Reactome Pathway Knowledgebase, Rhea, ChEBI, NCBI's Conserved Domain Database (CDD) etc. are available for the gene prediction in functional annotation (Fitihamlak and Jung, 2020).

Prokka which is a Unix-based command line software can be used for the rapid annotation of prokaryotic genomes while identifying the genomic features within contigs using external feature prediction tools, such as RNAmmer and Prodigal (Seemann, 2014). Simultaneously, for bacterial and archaeal genome annotation, Rapid Annotations using Subsystems Technology (RAST) is a fully automated pipeline with high accuracy, consistency and completeness utilizing a library of subsystems (Aziz *et al*., 2008).

### 1.6 Importance of studying *V. nigripulchritudo* genome and virulence

As the bacteria from the *Vibrio* genus are autochthonous to aquatic environments and ubiquitous in aquaculture production systems, they may share many mechanisms to cause infectious diseases in cultured organisms. Among them, *V. nigripulchritudo* is considered as an emerging pathogen causing the winter vibriosis syndrome 93, summer syndrome and associated vibriosis disease in penaeid shrimps creating mass mortalities.

To prevent and control the vibriosis in aquaculture production systems, it is necessary to obtain a better understanding of genomic background of the *V. nigripulchritudo* with respect to their pathogenicity and virulence. Also. The host-pathogen interaction is another importance to get a better insight about the disease.

There are very few scientific publications in the literature on genomic characterization and identification of *V. nigripulchritudo.* Therefore, it is a critical need to study the whole genome sequence of the isolated strains of *V. nigripulchritudo* to fill this information gap while providing a proper knowledge on the virulence genes responsible for their pathogenicity.

A new revolution has begun on bacterial diseases with the onset of bacterial whole genome sequencing for the discovery of the virulence related genes involved in pathogenicity of respective microbial pathogens. Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. Therefore, the study of genes and their functions, host-pathogen interactions and comparative genomics with other related species would allow a complete understanding on pathogenicity and virulence of *V. nigripulchritudo.* Concurrently, the information based on the whole genome characterization of *V. nigripulchritudo* would help to identify the preventive and controlling measures of the vibriosis disease of penaeid shrimps which would help to secure the seafood production.

### 1.7 Objectives of this study

The present study is aimed to get a better insight about the whole genome characteristics and the virulence related genes of the isolated strains of the *V. nigripulchritudo* collected from the white leg shrimp (*P. vannamei*) of Japan infected by vibriosis. The overall results have paramount validity in understanding disease development, controlling, and preventing methods of *V. nigripulchritudo*.

Specific objectives of this study are,

1. To isolate and identify the causative agents of *V. nigripulchritudo* from vibriosis infected white leg shrimps *P. vannamei*
2. To study whole genome characteristics of the isolated strains of *V. nigripulchritudo*
3. To identify and characterize virulence related genes of isolated *V. nigripulchritudo* while assessing their role on pathogenesis for penaeid shrimps

### 1.8 Outline of the study

The remaining parts of the thesis are indicated as follows.

Chapter 2 provides information on the identification and confirmation of the isolated V. nigripulchritudo from diseased white leg shrimp *P. vannamei* by focusing on the PCR amplification with 16S rRNA and hemolysin genes along with the Sanger sequencing and agglutination tests with polyclonal antibodies. Also, it includes about the study on host-pathogen relationship using the injection and immersion challenge tests conducted with Kuruma shrimp *P. japonicus*.

Chapter 3 explains about the use of whole genome sequencing with Illumina and Nanopore to study about the genomic features of the isolated strains of *V. nigripulchritudo*. It focuses mainly on the presence of virulence genes, prophages, genomic islands which are involved in the pathogenesis. Also, it describes about the information on orthologous genes and the phylogenetic relationship among the isolated *V. nigripulchritudo* along with two reference genomes of *V. nigripulchritudo.*

Chapter 04 provides a general conclusion on the identification and confirmation of the isolated *V. nigripulchritudo* along with the genomic characterization consisted with the presence of

virulence related genes, prophages, genomic islands etc. for understanding disease development, controlling, and preventing methods of *V. nigripulchritudo*.

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# Chapter 2

## Identification of causative agent of the isolated bacteria from the vibriosis infected the white leg shrimps *P. vannamei* and study on the pathogenicity of the isolated bacterial strains

### 2.1 Abstract

*Vibrio nigripulchritudo* is a halophilic gram negative, oxidative positive and O/129 sensitive bacteria which was firstly isolated in New Caledonia in 1995 from diseased blue shrimp *Penaeus stylirostris*. First reported event of mass mortality of Kuruma shrimp *P. japonicus* due to *V. nigripulchritudo* infection in Japan was occurred in 2005. We isolated the *V. nigripulchritudo* from a mass mortality event of white leg shrimps *P. vannamei* occurred in a closed marine aquarium in the Tokyo University of Marine Science and Technology, Tokyo, Japan. For the identification of the causative agent of the bacteria, molecular diagnostic methods including PCR amplification with 16S rRNA and hemolysin primers along with the Sanger sequencing and the use of polyclonal antibodies for agglutination test were used in this study. Simultaneously, standard challenge tests were conducted to determine the pathogenicity of the isolated bacterial strains for Kuruma shrimp *P. japonicus* through injection and infection challenge models.

After commencing the molecular diagnostics, all the isolated bacterial strains from the diseased shrimps were identified and confirmed as *V. nigripulchritudo* based on the 16SrRNA and hemolysin genes. The obtained sequence data from Sanger sequencing aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn) showed that all the isolated strains of the bacteria (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3) are having the highest homology with the available reference genomes of *V. nigripulchritudo* in Genbank database. Concurrently, results of the challenge tests showed that the isolated strains were pathogenically virulent for Kuruma shrimp *P. japonicus* in both immersion and injection infection indicating that the disease outbreaks due to *V. nigripulchritudo* could be able to cause severe damage to shrimp farming industry.

**Key words:** *Vibrio nigripulchritudo*, 16S rRNA, hemolysin, Sanger sequencing, pathogenicity

## 2.2 Introduction

*Vibrio nigripulchritudo* classified under family Vibrionaceae. is a halophilic gram negative, oxidative positive and O/129 sensitive bacteria which can be grown in a temperature range from 20-30 $^{0}$C (optimum temperature: 25 °C) and a salinity range of 3-5 ppt (Sakai *et al*., 2007). *V. nigripulchritudo* was firstly isolated in New Caledonia in 1995 from diseased blue shrimp *Penaeus stylirostris* affected by winter vibriosis syndrome 93 (Costa *et al*., 1998) followed by a second isolation in 1997 causing "Summer Syndrome", an acute systemic vibriosis with mass mortalities of cultured shrimps. (Goarant *et al*.,2000). First reported event of mass mortality of Kuruma shrimp *P. japonicus* due to *V. nigripulchritudo* infection in Japan was occurred in 2005 (Sakai *et al*., 2007).

For the conventional therapy of the vibriosis disease and for proper epidemiological characterization, rapid, sensitive, and specific identification and characterization of the causative organism is required. The use of proper culture medium for presumptive identification, biochemical identification, serological identification using monoclonal antibodies and identification using molecular methods can be applicable for the identification of *V. nigripulchritudo.*

Use of proper culture medium for presumptive identification can be applied at the initial stage of the identification process. Colonies of *V. nigripulchritudo* on brain heart infusion agar (5% NaCl) appear to be low convex, entire, circular, semi-transparent cream-colored colonies (Costa *et al*., 1998) while producing light greenish color colonies in Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar.

For the identification of *Vibrio* sp. having similar phenotypical and biochemical and properties, use of polyclonal antibodies prepared against sodium azide- killed cells can be applied as a rapid and specific method due to their high specificity. The use of polyclonal antibodies is widely adapted in detection and identification of *Vibrio* sp. to get faster results in less expensive manner (Wangman *et al*., 2021). Therefore, it can also be used to identify the causative strains of *V. nigripulchritudo.*

Molecular detection methods have been developed over the last decade to get more accurate and faster results as the conventional microbiological methods of detection, identification and characterization of *Vibrio* pathogens require time and frequently create false-negative/positive results (Gdoura *et al*., 2016). Therefore, for identification and typing of *Vibrio* sp. a various

types of molecular methods have been used with higher discriminatory power and higher reproducibility (Gomez *et al*., 2009).

16S rRNA gene sequence is widely used as a common housekeeping genetic marker to study the bacterial phylogeny and taxonomy. Due to the presence of 16S rRNA in almost all bacteria, consistent nature of the gene over time, and large enough size (1500 bp) for informatic purposes, 16S rRNA gene sequence is considered as one of the mostly used sequences to gain knowledge on the identification of species and genus of isolates (Janda and Abbott, 2007). According to Sakai *et al*. (2007), for the identification of *V. nigripulchritudo* isolated from Japan in 2005, the 16S rRNA sequence was used, and it gave the highest identity (99%) with the available *V. nigripulchritudo* ATCC27043 showing identical patterns in DNA hybridization (Gene bank accession number X74717).

Concurrently, As stated by Mizuno *et al.* (2019), hemolysin is one of the major pathogenic factors among *Vibrio* species indicating hemolytic activity against erythrocytes. It has been found that, virulent strains of most of the *Vibrio* sp. are able to produce the thermostable direct hemolysin (TDH) and/or the TDH-related hemolysin (TRH) and both of which are considered as molecular markers for the determining the pathogenicity of the species (Saito *et al*., 2015).

For a better understanding of host-microbe interactions, the studied organisms must be cultured in fully controlled conditions (Marques *et al*., 2006). Hence, a standardized model culture system with gnotobiotically cultured test organisms should be used for yielding predictable and reproducible results to study the role of bacteria in the culture of test organisms. To investigate the capability and the virulence of *V. nigripulchritudo*, a standardized challenge test with Kuruma shrimp *P. japonicus* was conducted with the isolated stains of *V. nigripulchritudo*.

In the present study, the isolated causative agents of the bacteria from diseased white leg shrimp *P. vannamei* are identified and confirmed as *V. nigripulchritudo* by PCR amplification with the 16S r RNA gene and hemolysin gene, agglutination test with monoclonal antibodies and Sanger sequence of 16S r RNA gene and hemolysin gene respectively. Simultaneously, standard challenge tests were conducted to determine the pathogenicity of the isolated bacterial strains for Kuruma shrimp *P. japonicus* through injection and infection challenge models.

## 2.3 Materials and Methods

### 2.3.1 Isolation of the causative agent of the bacteria

The bacterial samples were isolated form the mass mortality occurred in the white leg shrimp *P. vannamei* in a closed marine aquarium at Tokyo University of Marine Science and Technology, Tokyo, Japan in 2018. The tissues (muscles, hepatopancreas, gills) of diseased shrimp were streaked in heart infusion (HI) agar supplemented with 2.5 % of NaCl to isolate single colonies of the causative agents. Five samples (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT--V. nig3, TUMSAT-V. nig4 and TUMSAT-V. nig5) isolated from different shrimps were used for further studies,

### 2.3.2 Identification and confirmation of the isolated strains of bacteria

#### 2.3.2.1 Bacterial culture preparation

To prepare the brain heart infusion (HI) agar plates, 2.5 g of brain heart infusion (Difco), 1.5 g of agar powder (Wako) and 2.5 g of sodium chloride were dissolved in 100mL of distilled water. After autoclaving the medium at 121 °C for 20 min, medium was dispensed into plates (20 mL/plate) and allowed for solidification under the laminar flow (SANYO MCV-710ATS). Bacterial strains after thawing from the glycerol stock (60 %) stored at -80 C were inoculated by sterilized loop in each plate. The inoculated plates were closed by polythene wrap and incubated (SANYO -MIR 154) upright for 24 hours at 25 °C.

Thiosulfate citrate bile salt sucrose (TCBS) Agar plates were used for verifying the identification of the isolated bacterial strains with selective media. The respective plates were prepared using 89 g of TCBS (Difco) suspended in 1 L of distilled water. After mixing it thoroughly, the medium was heated with frequent agitation and boiled for 01 minute to completely dissolve the powder. After cooling the medium to 45- 50 °C, medium was dispensed into plates (20 mL/plate) and allowed for solidification under the laminar flow. The bacterial strains after thawing from the glycerol stock (60 %) stored at -80 °C were inoculated by sterilized loop in each plate. The inoculated plates were closed by polythene wrap and incubated upright for 24 hours at 25 °C.

To prepare the brain heart infusion culture broth, 2.5 g of brain heart infusion (Difco), and 2.5 g of sodium chloride were dissolved in 100mL of distilled water. After autoclaving the medium at 121 °C for 20 min, the bacterial colony grown in HI agar plates was inoculated in 5mL of HI

broth in 15 mL falcon tubes. The inoculated samples were incubated for 24 hours at 25 °C in shaker (BIO shaker -BR 40LF).

#### 2.3.2.2 PCR amplification with 16S rRNA and hemolysin primers

**DNA template preparation**

The colony Polymerase Chain Reaction (PCR) was used for the amplification of the isolated bacterial strains with 16S rRNA and hemolysin primers as described by Walch *et al*. (2016) with some modifications. A single colony from the respective HI agar plate was taken by a sterilized toothpick and kept into a PCR tube after mixing thoroughly. After adding 10 µL of distilled water, the samples were boiled in 95 °C for 10 min in thermal cycler (Applied Bio Systems- MiniAmp, USA).

**PCR amplification with 16S rRNA primers**

The amplification of isolated bacterial DNA was carried out using the primer pair 16S rRNA (F) and 16S rRNA (R). PCR was conducted in the thermal cycler (Applied Bio Systems-MiniAmp, USA) in a 20 µL volume reaction tube containing 2 µL of ten-fold buffer (15 mM MgCl2, 100 mMTrisHCl, 500 mM KCl), 0.4 µL of each primer (Eurofin, Japan) (Table 2.1), 1.6µL for each dNTP (Takara. Japan), and 0.1 µL of Ex Taq DNA polymerase (Takara. Japan) with 14.5 µL of distilled water and 1 µL of the DNA template. The amplification conditions were 5 min of initial denaturation at 85 °C, followed by 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, and a final extension step of 72 °C for 5 min.

**PCR amplification with hemolysin primers**

Another PCR amplification was conducted with hemolysin primer pair Hly (F) and Hly (R) designed for *V. nigripulchritudo*, *V. penaeicida*, *V. campbellii* respectively (Table 2.1). PCR was performed in 20 µL volume reaction containing 2 µL of ten-fold buffer (15 mM MgCl2, 100 mM TrisHCl, 500 mM KCl), 0.4 µL of each primer (Eurofin, Japan), 1.6µL for each dNTP (Takara. Japan), and 0.1 µL of Ex Taq DNA polymerase (Takara. Japan), with 14.5 µL of distilled water and 1 µL of the DNA template using thermal cycler (Applied Bio Systems-MiniAmp, USA). The amplification conditions were 5 min of initial denaturation at 95 ºC, followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, and a final extension step of 72 °C for 5 min.

Another PCR amplification was conducted with hemolysin primer pair Hly (F) and Hly (R) designed for *V. nigripulchritudo* for the isolated *V. nigripulchritudo* strains and the available

other *Vibrio* sp. as *V. lentus*, *V. alginolyticus, V, harveyi*, *V, coralliilyticus*, *V. olivae*, *V. hyugaensis*, *V. sinaloensis*, *V. penaeicida,* followed by a control. PCR was performed in 20 µL volume reaction containing 2 µL of ten-fold buffer (15 mM $MgCl_2$, 100 mMTrisHCl, 500 mM KCl), 0.4 µL of each primer (Eurofin, Japan), 1.6µL for each dNTP (Takara. Japan), and 0.1 µL of Ex Taq DNA polymerase (Takara. Japan), with 14.5 µL of distilled water and 1 µL of the DNA template using thermal cycler (Applied Bio Systems- MiniAmp, USA). The amplification conditions were 5 min of initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, and a final extension step of 72 °C for 5 min.

**Visualization of the PCR products**

3 µL of PCR product from each reaction were mixed with 1µL loading dye (six-fold diluted) and electrophoresed in a 1% (w/v) agarose gel with 1x Tris-acetate-EDTA (TAE) with 10 µL/L ethidium bromide for 15 min in 100V and visualized the PCR products with ultraviolet light using image saver (AE-6905C ATTO, Japan).

**Clean the PCR products prior to sequencing**

To clean the obtained PCR products prior to sequencing, ExoSAP-IT® (Applied Biosystems, USA), based on Exonuclease I and Shrimp Alkaline Phosphatase, is used as described by Bell (2008). 0.1 µL of ExoSAP-IT mixed with 3.9 µL of distilled water was directly added to 10 µL of the obtained PCR products to degrade the unconsumed primers and dephosphorylate dNTPs. Treatment was carried out for 30 min at 37°C, followed by a 15 min incubation at 80°C in MiniAmp thermal cycler (Applied Bio Systems, USA) to perform the downstream sequencing of PCR products.

**2.3.2.3 Sanger sequencing with 16S r RNA and hemolysin gene**

**Sample preparation**

Sanger sequencing of the isolated strains of bacteria with 16S r RNA and hemolysin gene was conducted according to the method described by Sanger *et al*. (1977) with modifications. The contents of the BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) and the primers (Table 2.1) were completely thawed and vortex for 2 to 3 sec before using. Two sets of PCR tubes were labeled as "forward" and "reverse" separately with the sample names. Each tube was added with 1 µL of BigDye™ Terminator v3.1, 3.5 µL of 5x sequencing buffer, 12.5 µL of deionized water (RNase/DNasefree), 1 µL of 16S r RNA primer (forward and reverse separately) followed by the 2µL of purified PCR product of the sample (ExoSAP-IT™ Express PCR Product Cleanup).

Another two sets of PCR tubes were prepared as "forward (F)" and "reverse (R)" separately with the sample names and added with the same components while changing the primer into hemolysin (forward and reverse separately). The amplification was carried out for both sample sets with 16S r RNA and hemolysin primers in thermal cycler with 1 min of initial denaturation at 96 °C, followed by 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min, and holding at 4 °C for further use.

**Purification of the sequencing products with ethanol/EDTA precipitation**

20 µL of PCR products of each sample (forward and reverse) were added into newly labelled set of 1.5 mL Eppendorf tubes. 10 µL of 125 mM EDTA and 30 µL of 99% ice cold ethanol were added to each sample tube. After centrifugation of the samples at 12000 rpm for 15 min at 4 °C, the supernatant was removed by pipetting. To the pellet, 100 µL of 75 % ice cold ethanol was added and centrifuged again at 15000 rpm for 5 min at 4 °C. After the removal of the supernatant completely, the product was dried at 60 °C for 02 hrs and air dried overnight.

**Resuspend purified sequencing products and run capillary electrophoresis**

Completely dried samples were resuspended in 10 µL of Hi-Di™ Formamide. After thoroughly vortex the samples for 10 sec, the samples were centrifuged for 10 sec at 1,00000 rpm. After that, samples were sequenced in 3130 XL Genetic Analyzer (Applied Biosystem, USA). Nucleotide sequences of 16S rRNA and hemolysin region from the isolated strains of *V. nigripulchritudo were* identified and homology analysis was performed by the nucleotide Basic Local Alignment Search Tool (BLASTn; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2.1: The base sequences of the primers used for the study

| Primer | Base sequence |
|---|---|
| 16S rRNA (forward) | 5'- CGTGCCAGCAGCCGCG – 3' |
| 16S rRNA (reverse) | 5' - GCCCGGGAACGTATTC – 3' |
| Hemolysin_ *V. nigripulchritudo* (forward) | CTAATAGCATCACACATGCGCTAG |
| Hemolysin_ *V. nigripulchritudo* (reverse) | ACCAGACCACCCACATCGAGCGTC |
| Hemolysin_ *V.penaeicida* (forward) | CCAATAGCATCACACATGCGCTAG |
| Hemolysin_ *V.penaeicida* (reverse) | ACCAGACCACCCACATCGAGCGTG |
| Hemolysin_ *V.campbellii* (forward) | CCAACTCCATTACTCATGGTATTGG |
| Hemolysin_ *V.campbellii* (reverse) | GCGTCAAACCACCAATATCCAGATT |

**2.3.2.4 Agglutination test with polyclonal antibodies**

**Production of the antiserum of *V. nigripulchritudo***

The anti-serum of *V. nigripulchritudo* was prepared according to the method described by Patil *et al*. (2013) with some modifications. *V. nigripulchritudo* were grown 2.5 % HI broth with 2.5 % NaCl for 24 hours under constant stirring condition at 25 °C at shaker ((BIO shaker -BR -40LF). Harvesting of the bacterial cells were done by centrifugation at 13,500 rpm and processed and deactivated with formalin (0.5%). After assessing the density of the bacterial cell, concentration was adjusted to $10^{10}$ CFU/ml. The suspension was stored at 4 °C till further use. The collected formalin killed cells of *V. nigripulchritudo* were sent to Eurofin, Japan to produce the antiserum respect to *V. nigripulchritudo*.

**Antibody titer test**

The antibody titer test is conducted to detect the presence and measure the amount of antibody in blood. Antibody titer test for the antiserum production of *V. nigripulchritudo* was conducted as described by Son and Taylor (2011) with some modifications. Bacterial culture was prepared in 2.5 % HI broth with 2.5 % NaCl for 24 hours under constant stirring condition at 25 °C.

In a round bottom 96 well plate labelled for sample and negative control, 50 µL of sterile PBS was added to each well. 50 µL of the respective anti serum sample was added into the first well and mixed thoroughly. 50 µL of the first well was pipetted and added into second well of the

same raw and mixed well. The process was repeated along the rows to the final wells to make a twofold serial dilution of the anti-serum across the row. After mixing the sample in the final well, 50 µL of the sample was pipetted and discarded.

10 µL of cultured *V. nigripulchritudo* was added into the each well of the first raw. The second row was remained without addition of the bacteria as a negative control. The plate was incubated at room temperature for overnight after covering with aluminum foil to protect it from light. The antibody titer was determined by observing the presence of coagulants at the following day.

**Agglutination test**

The agglutination test is conducted to find out whether the antigens are capable of binding to a specific antibody. After determination of the antibody titer, the obtained antiserum was diluted 8 times to perform the agglutination test. In a clean glass slide, 50 µL of each pre immune serum and after immune serum were added respectively. After taking a single colony grown in HI agar plate, it was mixed with the antiserum samples separately. After keeping the slides for 1 min in room temperature, the agglutination was checked. For agglutination test, *V. parahaemolyticus* was used as a positive controller along with the experimental samples of *V. nigripulchritudo*.

### 2.3.3 Assessment of virulence in Kuruma shrimp *Penaeus japonicus*

#### 2.3.3.1 Bacterial sample preparation

For the challenge tests, concentration of the bacteria was checked before starting the experiment by using the colony counting method. 90 µL of artificial sea water was added to 12 wells of a 96 well plate. 10 µL of the bacteria cultured in HI broth was added and mixed well. 10 µL of the first well was pipetted and added into second well of the same raw and mixed well. The process was repeated along the rows to the final wells to make a tenfold serial dilution of the bacteria. After mixing the sample in the final well, 10 µL of the sample was pipetted and discarded.

5 µL from each well was added to HI agar plate and TCBS agar plate which were divided into 6 portions for representing the different bacterial concentrations. After keeping for 30 min in room temperature, the plates were incubated for 24 hours at 25 °C in incubator (SANYO -MIR 154). The number of colonies were count in the following day in each dilution series and the concentration of the inoculated sample was calculated using the average values.

#### 2.3.3.2 Immersion challenge test

15 of juvenile Kuruma shrimp *P. japonicus* (4- 5 g) were kept in four 10 L glass tanks supplied with sea water separately for the immersion challenge test. Each tank was immersed with a bacterial concentration of $10^6$ CFU/mL, $10^7$ CFU/mL, and $10^8$ CFU/mL of the isolated strain of *V. nigripulchritudo* TUMSAT-V. nig1 respectively followed by a control group. The aeration was changed after 6 hours of the immersion. The experimental shrimp were fed daily and monitored. The dead shrimps were collected, and the mortality was recorded daily.

#### 2.3.3.2 Injection challenge test

For the injection challenge test, 15 shrimps were used for each experimental challenge doses of $10^2$ CFU/mL, $10^3$ CFU/mL, and $10^4$ CFU/mL. Experimental shrimps were kept in four 10 L glass tanks supplied with sea water separately. The shrimps were intraperitoneally injected with 50 µL of each dose emulsified in artificial sea water. The control group of the shrimps was intraperitoneally injected with 50 µL of artificial sea water. The mortality rate was observed daily over a period of 10 days.

The dead shrimps from both immersion and injection challenge tests were collected. Their hepatopancreas and muscles were streaked in HI and TCBS plates and incubated for overnight at 25 °C to check the presence of the bacteria. After re isolation of the bacteria, PCR amplifications were conducted using 16S r RNA and hemolysin primers to confirm the bacterial reisolates.

## 2.4 Results

### 2.4.1 Identification and confirmation of the isolated bacterial strain

#### 2.4.1.1 PCR amplification with 16S rRNA and hemolysin primers

Results of the PCR amplification with 16S rRNA showed that all the isolated samples were having the respective bands in gel after electrophoresed with 1% (w/v) agarose gel with 1x Tris-acetate-EDTA (TAE) with 10 µL/L ethidium bromide for 15 min in 100V (Figure 2.1). The results indicated that the isolated bacterial strains from the moribund white leg shrimp *P. vannamei* are *V. nigripulchritudo* as the strains got positive amplification results with respect to 16S rRNA primer designed for *V. nigripulchritudo*.

Simultaneously, the PCR amplification conducted with hemolysin primers; Hly (F) and Hly (R) designed for *V. nigripulchritudo*, *V. penaeicida*, and *V. campbellii* showed the positive results

only for the isolated bacterial samples added with the Hly primer designed for *V. nigripulchritudo* (Figure 2.2). The rest of the isolated bacterial samples added the Hly primer designed for with *V. penaeicida* and *V. campbellii* did not show the amplified bands on the gel. The results indicated that the isolated strain is *V. nigripulchritudo* depending on the PCR amplification results of Hly primer.

The results of the PCR amplification with the hemolysin primer for the isolated bacterial strains of *V. nigripulchritudo* and other *Vibrio* species have shown that the positive amplification results only for the isolated strains as *V. nigripulchritudo*. The rest of the other *Vibrio* species did not show any positive amplification with the hemolysin primer designed for *V. nigripulchritudo* (Figure 2.3).

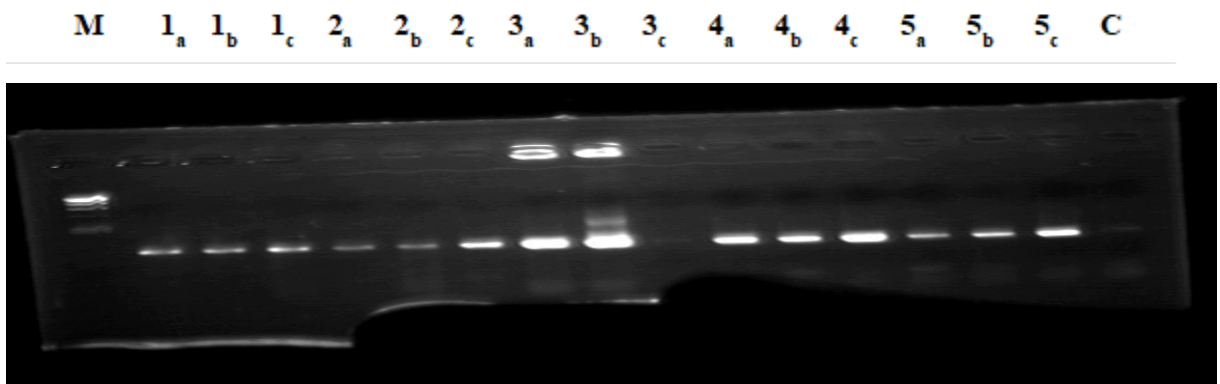


Figure 2.1: PCR amplification of 16S rRNA gene (M: λ HindIII ladder, lane 2-15: PCR products of bacterial strains; 1: TUMSAT-V. nig1, 2: TUMSAT-V. nig2, 3: TUMSAT-V. nig3, 4: TUMSAT-V. nig4, 5: TUMSAT-V. nig5, C: control; a,b,c : respective replicates)

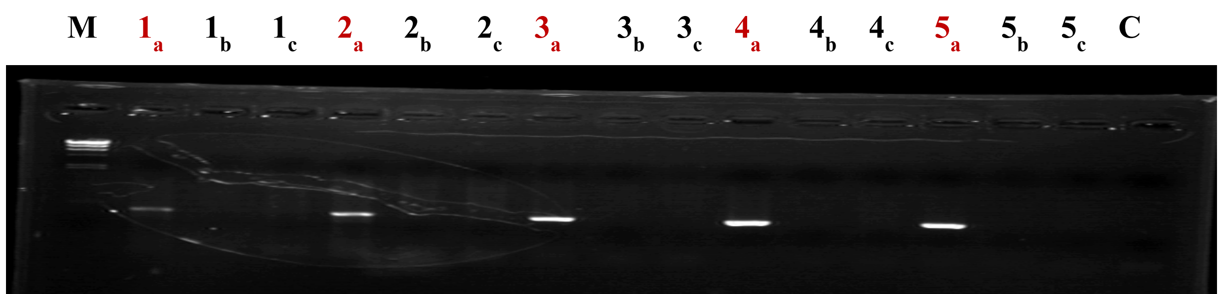


Figure 2.2: PCR amplification of Hemolysin gene (M: λ HindIII ladder, lane 2-15: PCR products of bacterial strains; 1: TUMSAT-V. nig1, 2: TUMSAT-V. nig2, 3: TUMSAT-V. nig3, 4: TUMSAT-V. nig4, 5: TUMSAT-V. nig5. a: *V.nigri*+ primer Hly(*V.nigri*), b : *V.nigri*+ primer Hly(*V.pen*), c: *V.nigri*+ primer Hly(*V. cam*), C: *V.pen*+ primer Hly(*V.nigri*)

Figure 2.3: PCR amplification of Hemolysin gene (M: λ HindIII ladder, lane 2-15: PCR products of bacterial strains; 1: TUMSAT-V. nig1, 2: TUMSAT-V. nig2, 3: TUMSAT-V. nig3, 4: *V. lentus*, 5: *V. alginolyticus* (1), 6: *V. alginolyticus* (2). 7: *V, harveyi* (1), 8: *V, harveyi* (2), 9: *V, coralliilyticus*, 10: *V. olivae*, 11: *V. hyugaensis*, 12: *V. sinaloensis*, 13: *V. penaeicida* (1), 14: *V. penaeicida* (2), 15: *V. penaeicida* (3), 16: control

**2.4.1.2 Sanger sequencing with 16S r RNA and hemolysin gene**

**Alignment with 16S rRNA gene**

The obtained sequence data amplified with 16S rRNA primer from Sanger sequencing aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn; https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that all the isolated strains of the bacteria (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3, and TUMSAT-V. nig4) are having the highest homology with the available reference genomes of *V. nigripulchritudo* available in gene bank. The isolated strain TUMSAT-V. nig1 is identified as *V. nigripulchritudo* which shows 99.9 % of percentage identity with *Vibrio nigripulchritudo* strain F77028 16S ribosomal RNA gene (Gene bank accession number: JF281755.1) with an E value of 0.0. The pairwise alignment view shows that the isolated TUMSAT- V. nig1 strain was aligned with F77028 16S ribosomal RNA gene with a coverage of 99.9 % with no mismatches (Figure 2.4).

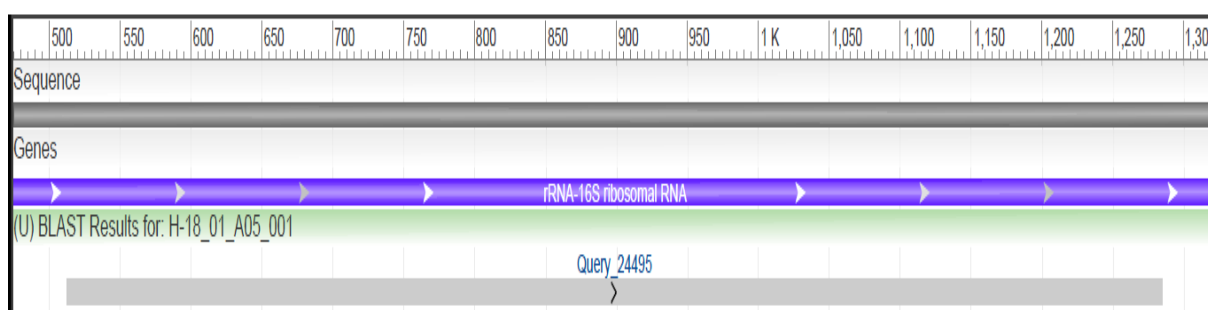


Figure 2.4: Pairwise alignment of isolated TUMSAT-V. nig1 with *Vibrio nigripulchritudo* strain F77028 16S ribosomal RNA gene, partial sequence (GenBank: JF281755.1)

The Blast Tree View with neighbor joining generated by BLASTn in NCBI showed that the isolated strain is phylogenetically related with the available genomes of *V. nigripulchritudo* showing the same evolutionary pathways (Figure 2.5).

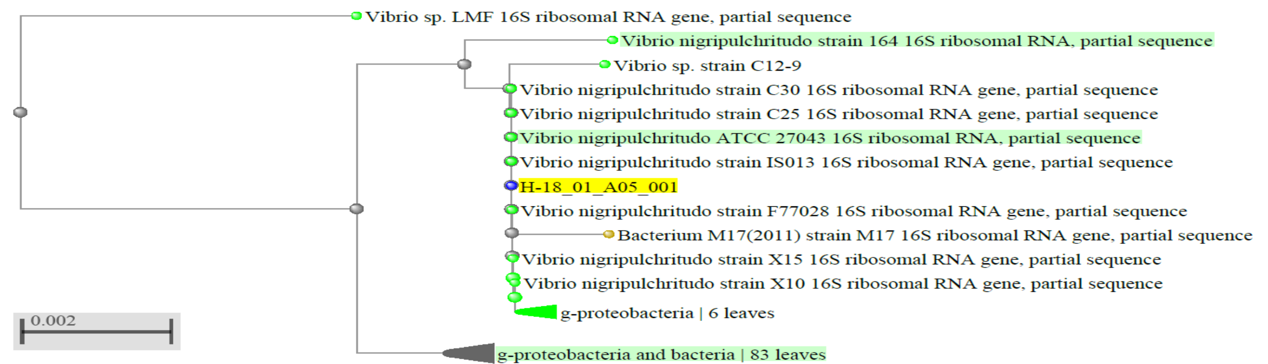


Figure 2.5: The Blast Tree View neighbor joining generated using BLASTn for the isolated strain of TUMSAT-V. nig1 with respect to 16S rRNA gene

Concurrently, for the isolated strain of TUMSAT-V. nig2, TUMSAT-V. nig3, and TUMSAT-V. nig4, we got the highest percentage identity with the same *Vibrio nigripulchritudo* strain F77028 16S ribosomal RNA gene, partial sequence (GenBank: JF281755.1) as 99.7 %, 100 % and 99.8 % respectively. Therefore, these results indicate that the isolated strains of *V. nigripulchritudo* are having a same origin and similar evolutionary background.

**Alignment with hemolysin gene**

The obtained sequence data amplified with hemolysin primer from Sanger sequencing aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn; https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that all the isolated strains of the bacteria (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3, and TUMSAT-V. nig4) are having the highest homology with the available reference genomes of *V. nigripulchritudo* available in gene bank. The isolated TUMSAT-V. nig1 strain was with the highest homology with *Vibrio nigripulchritudo* TUMSAT-TG-2018 having 99.2 % identity and E value of 0.0 (gene bank accession number: AP024087.1). The pairwise alignment view shows that the isolated TUMSAT-V. nig1 strain was aligned with *Vibrio nigripulchritudo* TUMSAT-TG-2018 with a coverage of 99.5 % with 2 mismatches (Figure 2.6). The Blast Tree View with neighbor joining generated by BLASTn in NCBI showed that the isolated strain is phylogenetically related with the available genomes of *V. nigripulchritudo* with respect to the base sequence of hemolysin gene (Figure 2.7).

Simultaneously, the isolated strains of TUMSAT-V. nig2, TUMSAT-V. nig3, and TUMSAT-V. nig4 are showing highest homology with the *Vibrio nigripulchritudo* SFn1 chromosome (gene bank accession number: FO203526.1) with percentage identities of 98.2 %, 98.4 %, and 98.7 % respectively with respect to the base alignment of hemolysin primer.

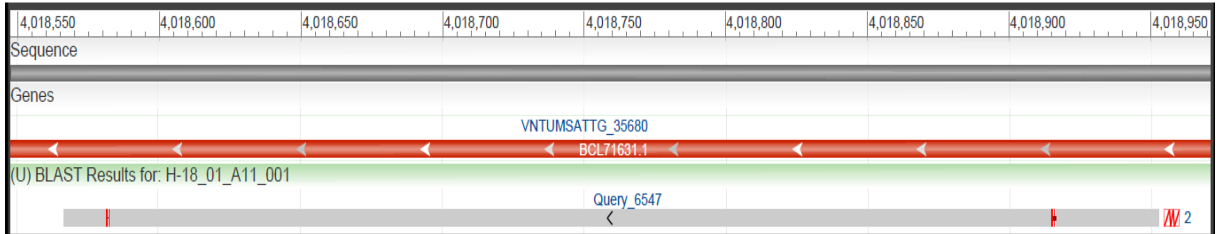


Figure 2.6: Pairwise alignment of isolated TUMSAT-V. nig1 with *Vibrio nigripulchritudo* TUMSAT-TG-2018 with respect to hemolysin gene (GenBank: AP024087.1)

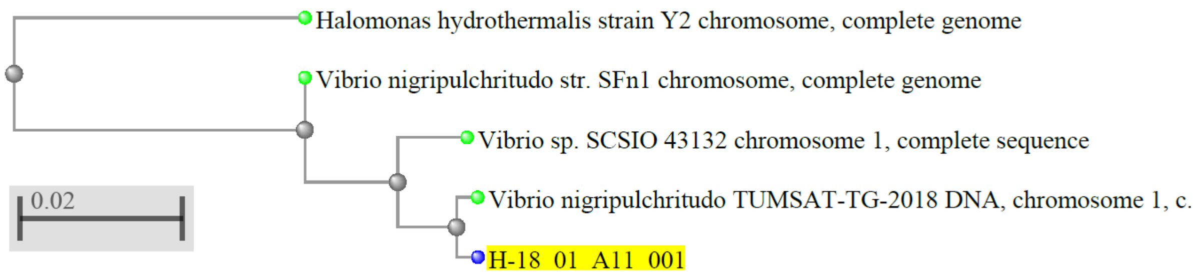


Figure 2.7: The Blast Tree View neighbor joining generated using BLASTn for the isolated strain of TUMSAT-V. nig1 with respect to hemolysin gene

### 2.4.1.3 Agglutination test with monoclonal antibodies

The antibody titer test was conducted to determine the presence and measure of the amount of antibody in blood. The titer is determined as the reciprocal of the highest dilution of the antibody/serum sample. After the serial dilution of the obtained antibody with PBS, it has been found that the last dilution of a serum sample that responds in the assay is the sample diluted as 1: 512 (Figure 2.8). Therefore, the antibody titer for the respective antibody designed using *V. nigripulchritudo* is determined as 512. Therefore, the respective diluted antibody samples were used for the agglutination test.

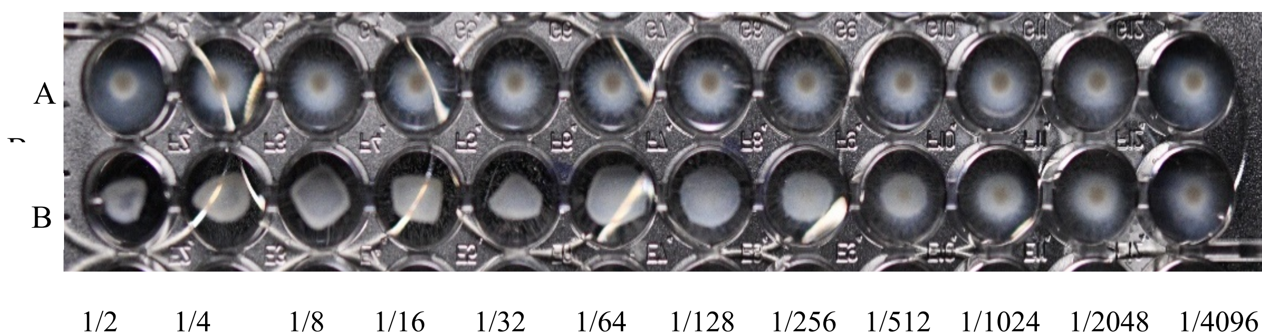


Figure 2.8: The 96 well plate with the antibody titer test after 24 hours with the serial dilution (A: negative control, B: the antibody sample treated with the isolated *V. nigripulchritudo* strain TUMSAT-V. nig1)

The results of the agglutination test showed that all the isolated bacterial strains have shown the positive result with the antiserum produced by using Formalin killed *V. nigripulchritudo*. Therefore, these results indicate the isolated bacterial strains are *V. nigripulchritudo* based on the antiserum positivity.

### 2.4.2 Assessment of virulence in Kuruma shrimp *Penaeus japonicus*

#### 2.4.2.1 Injection challenge test

After injecting the experimental Kuruma shrimp *P. japonicus*, the highest mortality was observed for the shrimps injected with $10^4$ CFU/mL, followed by $10^3$ CFU/mL and $10^2$ CFU/mL. At the end of the experiment, the percentage survival of the infected shrimps was 6.67%, 20.0 % and 26.7 % for the doses of $10^4$ CFU/mL, $10^3$ CFU/mL, and $10^2$ CFU/mL respectively (Figure 2.9).

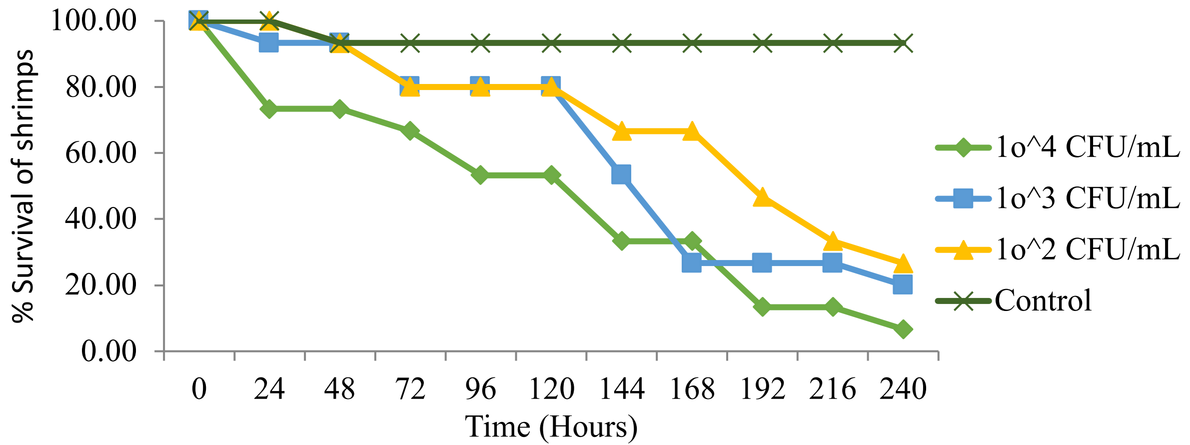


Figure 2.9: Percentage Survival of Kuruma shrimp (*P. japonicus*) infected with *V. nigripulchritudo*- TUMSAT- V. nig1 through injection infection with the doses of $10^4$ CFU/mL, $10^3$ CFU/mL, and $10^2$ CFU/mL

**2.4.2.1 I Immersion challenge test**

After challenging the experimental Kuruma shrimp *P. japonicus* with the doses of $10^6$ CFU/mL, $10^7$ CFU/mL, and $10^8$ CFU/mL, the highest mortality was observed for the shrimps immersed with $10^8$ CFU/mL while the lowest mortality was in $10^6$ CFU/mL (Figure 2.10). After 24 hours of the post infection, sudden drop of the survival was observed in the shrimps infected with $10^8$ CFU/mL later followed by the other doses with comparatively a lower rate.

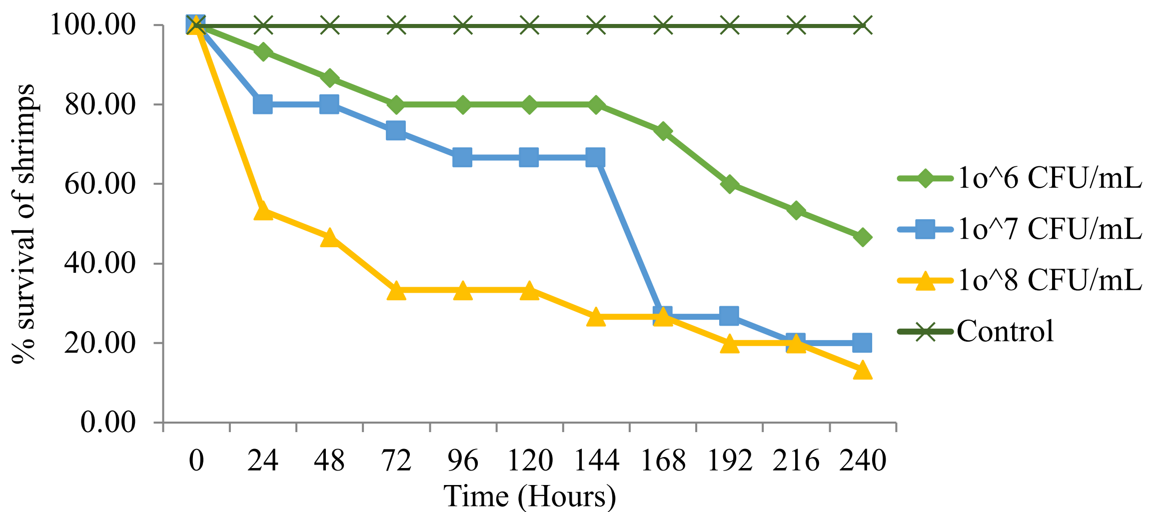


Figure 2.10: Percentage Survival of Kuruma shrimp (*P. japonicus*) infected with *V. nigripulchritudo* TUMSAT- V. nig1 through immersion infection with the doses of $10^6$ CFU/mL, $10^7$ CFU/mL, and $10^8$ CFU/mL

### 2.5 Discussion

Intending to acknowledge the pathogenic processes involved in a particular disease and to treat and prevent the disease successfully, the isolation and the identification of the respective etiological agent is highly required. Compared with the other bacterial species, *Vibrio* sp. are easier to isolate from their environment after provisioning of their specific growth factors. For the isolation of the *Vibrio* sp., the media must be supplemented with salt (NaCl) to equivalent to the natural marine environment of *Vibrio* sp. Therefore, HI medium supplemented with 2.5% was used for the isolation of *V. nigripulchritudo* in this study. Also, the TCBS agar was also used as the traditional specific media developed for culturing of *Vibrio* sp. As stated by Thompson *et al*. (2004), strains which can ferment glucose will form yellow colonies on TCBS medium while other strains will produce green colonies. The isolated bacterial species also produced light green color colonies in TCBS agar medium. *V. nigripulchritudo* is a halophilic gram negative, oxidative positive and O/129 sensitive bacteria which can be grown in a temperature range from 20-30 °C (optimum temperature: 25 °C) and a salinity range of 3-5 ppt (Sakai *et al*., 2007). Using both TCBS and HI agar medium supplemented with 2.5% salt, we were able to isolate *V. nigripulchritudo* as pure and healthy colonies after an overnight incubation in the optimum temperature of 25 °C.

Because of great variations of the phenotypic profiling of the *Vibrio* species even within the same species, the phenotypic identification is problematic. Also, the phenotypic and biochemical identification of the isolated *Vibrio* sp. are labor intensive, requiring many biochemical and physiological tests which were frequently expressed with false negative/ positive results (Vandenberghe *et al*., 2003). Therefore, to improve the diagnosis of *Vibrio* infections, rapid and specific methods are required for the identification of the causative strains of bacteria.

Polyclonal antibodies prepared against formalin or heat killed cells can be applied as a rapid and specific method to identify different *Vibrio* sp. having similar phenotypical, and biochemical properties as this method is highly specific for those species. As the polyclonal antibodies provide specific and reproducible immunological assay for rapid and accurate diagnosis of different types of infectious diseases, application of agglutination test with antibodies in the detection of *Vibrio* sp. is popular among scientists (Wangman *et al*., 2021). Therefore, this method can also be applicable to identify the causative strains of *V. nigripulchritudo*. In our study, the isolated strains of the bacteria gave positive identification as

*V. nigripulchritudo* when treated with the anti-serum produced from shrimps fed with formalin killed cells of *V. nigripulchritudo*.

The use of monoclonal antibody better suited for identification purposes than polyclonal antibodies due to their homogeneity, high specificity to a single epitope, their low degree of cross-reactivity although the cost of production is higher. However, we have used the polyclonal antibodies produced from shrimps fed with formalin killed cells of *V. nigripulchritudo* as the sensitivity is higher for low quantity of proteins. Therefore, to minimize the recognition of the multiple epitopes on the target antigen, the diluted samples of the respective anti serum was used for the identification by determining the antibody titer of the anti-serum. It would help to minimize the possibility of getting false positive results.

To overcome the challenges of conventional methods, different types of molecular detection methods have been developed over the last decade to get more accurate and faster results (Gdoura *et al*., 2016). For this study, we used PCR amplification and Sanger sequencing with 16S rRNA and hemolysin primers and agglutination test with monoclonal antibodies to identify the isolated strains of bacteria from the diseased white leg shrimp *P. vannamei*.

To study the bacteria phylogeny and taxonomy, use of 16S rRNA gene sequences is the most common housekeeping genetic marker due to its presence in almost all bacteria without changing its function over time and having an appropriate enough size (1500 bp) for informatics purposes (Janda and Abbott, 2007). Therefore, we also used the 16S rRNA sequencing for the identification of the isolated bacterial strain. All the isolated strain were identified as *V. nigripulchritudo* based on the 16S rRNA sequence data after aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn). The highest homology was shown to the *Vibrio nigripulchritudo* strain F77028 16S ribosomal RNA gene, partial sequence (GenBank: JF281755.1) for all the isolated strains. Therefore, these results indicate that the isolated strains of *V. nigripulchritudo* are having a same origin and similar evolutionary background. The obtained results were corresponded with the finding of the Sakai *et al*. (2007), for the identification of *V. nigripulchritudo* isolated from Japan in 2005. In there also, the 16S rRNA sequence was used, and it gave the highest identity (99%) with the available *V. nigripulchritudo* ATCC27043 showing identical patterns in DNA hybridization (Gene bank accession number X74717).

Although the 16S rRNA sequencing is considered as one of the most attractive potential parameters for species identification, some drawbacks are identified in this technique. As stated by Bosshard *et al*. (2006), the 16S rRNA sequencing technique has low phylogenetic power at

the species level and poor discriminatory power for some genera. Also, as the public available databases are used for the identification of new species, the overall quality of nucleotide sequences deposited in public databases must be thoroughly studied. Therefore, advancements in whole sequencing technologies are developed with greater numbers of sequencing reads at much lower costs for the characterization of microbial communities with high discriminating power (Degnan and Ochman, 2012) to explore the emergence of pathogens yielding novel insights into pathogenic evolution.

Hemolysin is one of the major pathogenic factors among *Vibrio* species indicating hemolytic activity against erythrocytes (Mizuno *et al.*,2019). Numerous *Vibrio* sp. are found with the presence of the hemolysin gene as one of the virulence factors in the species of *V. anguillarum* (Hirono *et al*., 1996; Rodkhum *et al., 2005*), *V. parahaemolyticus* (Nishibuchi and Karper, 1995), *V. vulnificus* (Yamamoto *et al*., 1990) etc. It has been found that, virulent strains of most of the *Vibrio* sp. are able to produce the thermostable direct hemolysin (TDH) and/or the TDH-related hemolysin (TRH) and both of which are considered as molecular markers for the determining the pathogenicity of the species (Saito *et al*., 2015). In our study, the isolated bacterial samples gave positive results when they were treated with hemolysin primer designed for *V. nigripulchritudo*. Also, the negative results were obtained when the samples were amplified with the hemolysin primer designed for *V. penaeicida* and *V. campbellii* indicating the specificity of the hemolysin gene in the respective species.

Also, the positive amplification was observed for the isolated *V. nigripulchritudo* while the negative results were obtained for the other *Vibrio* species treated with the hemolysin primer. The detection of the toxin genes encoding the thermostable direct or thermolabile hemolysin by separately designed primers would be useful as one of the simplest and most frequently used as a rapid and specific tool in identification of the *Vibrio* related species. Therefore, we can suggest that the use of the hemolysin primer is more suitable for the identification of the closely related *Vibrio* sp. as the respective hemolysin gene is highly conserved among the species.

To check the pathogenicity of an isolated strain, the use of standard challenge models is highly needed for a better understanding of host-microbe interactions (Marques *et al*., 2006). Hence, a standardized model culture system with gnotobiotically cultured test organisms should be used for yielding predictable and reproducible results to study the role of bacteria in the culture of test organisms. To investigate the capability and the virulence of *V. nigripulchritudo*, a standardized challenge test with Kuruma shrimp *P. japonicus* was conducted with the isolated stains of *V. nigripulchritudo* showing the pathogenicity of the isolated strain TUMSAT V. nig1.

The findings of the challenge tests would suggest that the isolated *V. nigripulchritudo* strain is pathogenically virulent for Kuruma shrimp *P. japonicus* in both injection and immersion challenge infections. It indicates that the disease outbreaks due to *V. nigripulchritudo* could be able to cause severe damage to shrimp farming industry. However, the dose specificity, strain specificity of the respective bacteria for different species of penaeid shrimps needed to be further analyzed to get a better insight about the pathogenicity.

## 2.6 Conclusion

The isolated bacterial strain from the diseased white leg shrimps *P. vannamei* were identified and confirmed as *V. nigripulchritudo* using the PCR amplification with 16s RNA and hemolysin primers. Specially in the case of amplification with hemolysin primer, the positive results were only obtained for the isolated strain added with hemolysin primer designed for *V. nigripulchritudo* indicating a higher specificity of hemolysin gene for the identification of *Vibrio* related species. The obtained sequence data from Sanger sequencing aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn) showed that all the isolated strains of the bacteria (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3) are having the highest homology with the available reference genomes of *V. nigripulchritudo* in Genbank database. Also, the isolated strain was identified as *V. nigripulchritudo* based on the agglutination test with respect to its antigen binding capability.

The present isolate showed high pathogenicity to Kuruma shrimp, *P. japonicus* in the immersion and injection experimental infections. It indicates that the disease outbreaks due to *V. nigripulchritudo* could be able to cause severe damage to shrimp farming industry. However, the dose specificity, strain specificity of the respective bacteria for different species of penaeid shrimps needed to be further analyzed to get a better insight about the pathogenicity.

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# Chapter 3

## Genomic characterization and identification of virulence related genes of isolated *V. nigripulchritudo*

### 3.1 Abstract

*Vibrio nigripulchritudo* is a halophilic gram negative, oxidative positive and O/129 sensitive bacteria which were classified under family Vibrionaceae. With the development of technological advances over the last two decades, a remarkable rising of whole genome sequencing technology has contributed to the determination of the genomic features of the organisms. Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. Therefore, the Illumina and Nanopore whole genome sequencing technology was used to create hybrid genomic assemblies as the long reads provide information on the genome structure while the short reads provide high base-level accuracy. The genome assembly of the isolated strains of *V. nigripulchritudo* has revealed that all the strains are occupied with two chromosomes (chromosome I and chromosome II) with the presence of either one or two plasmids in their genomes.

Numerous virulence related genes are present in either chromosome I or chromosome II related with the adherence, antiphagocytosis, chemotaxis and motility, iron uptake, quorum sensing, secretion systems and toxins causing the pathogenicity in penaeid shrimps. The presence of prophages, antibiotic resistance genes and genomic islands indicating that this bacterium can acquire new genetic information through horizontal gene transfer which has a significant role in bacterial evolution and affects pathogenesis. The comparative genomic analysis based on whole-genome proteome data of five *V. nigripulchritudo* genomes (three isolated strains with two reference genomes of *V. nigripulchritudo*) showed that the resulting tree split the *V. nigripulchritudo* strains into three clusters. The higher evolutionary homology is observed in the TUMSAT-V. nig1 and TUMSAT-TG-2018 and TUMSAT-V. nig2 and TUMSAT-V. nig3 respectively while the SFn1-2013 isolated from New Caledonia split into a separate cluster.

With the findings of the genome assembly and annotated genomic features related with the virulence factors and mobile genetic elements, the present study would be important for the prevention and the control of the spread of the vibriosis disease in penaeid shrimps.

**Key words:** *V. nigripulchritudo*, whole genome sequencing, virulence genes, mobile genetic elements, comparative genomic analysis

## 3.2 Introduction

*Vibrio nigripulchritudo* halophilic gram negative, oxidative positive and O/129 sensitive bacteria which can be grown in a temperature range from 20-30 °C (optimum temperature: 25 °C) and a salinity range of 3-5 ppt (Sakai *et al*., 2007). They are classified under family Vibrionaceae. The first isolation of the *V. nigripulchritudo* was occurred in New Caledonia in 1995 from diseased blue shrimp *Penaeus stylirostris* which was found in two adjoining farms suggesting a geographically restricted phenomenon. However, after the second isolation where it is far away from the first isolated area, the geographically restricted phenomena were rejected and hereafter, the disease was considered as "Summer Syndrome", an acute systemic vibriosis with a highly stereotyped epidemiology causing high mortalities in cultured shrimps (Goarant *et al*.,2000). First reported event of mass mortality of Kuruma shrimp *P. japonicus* due to *V. nigripulchritudo* infection in Japan was occurred in 2005 (Sakai *et al*., 2007). Higher mortalities were associated around 27 °C in July to August. Hence, it is discussed that the higher water temperature may enhance the severity of the disease caused by *V. nigripulchritudo*. Therefore, this disease must be seriously addressed in present along with the global warming with the consideration of the genomic characterization and virulence related genes involved in their pathogenicity.

The complete genetic information of an organism or a cell is called as the genome where the single or double stranded nucleic acids (DNA and RNA) stored the information in a linear or circular sequence. Efficient technologies were developed to increase the accuracy, throughput, and sequencing speed to precisely determine the genome sequence of a particular organism. With the development of technological advances over the last two decades, a remarkable rising of DNA sequencing technology has contributed to the new generation of sequencing methods which were targeted to complement and replace the Sanger sequencing eventually (Quail *et al*., 2012). The new technology was named as either next-generation sequencing (NGS) or massively parallel sequencing (MPS) with a wide diversity of approaches. These techniques can be used to generate massive amounts of data in a faster and cost-effective way. At present, numerous brands are found as Illumina, Ion Torrent (Thermo Fischer Scientific), BGI Genomics, PacBio and Oxford Nanopore Technologies in the global NGS market (Pereira *et al*., 2020).

The sequencing techniques are used to compare the gene content, genomic organization, and gene expression within species of multiple strains. Therefore, the comparative study of closely

related genomes improves our understanding about the evolutionary processes involved in the emergence of new infectious diseases.

Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. A new revolution has begun on bacterial diseases with the onset of bacterial whole genome sequencing for the discovery of the virulence related genes involved in pathogenicity of respective microbial pathogens. Therefore, the study of genes and their functions, host-pathogen interactions and comparative genomics with other related species would allow a complete understanding on pathogenicity and virulence of *V. nigripulchritudo.*

### 3.3 Materials and methods

#### 3.3.1 Genomic DNA extraction

For the extraction of the genomic DNA from the isolated bacteria, the NucleoBond ® AG columns with NucleoBond Buffer set III were used according to the manufacturer's guidelines. For that, the isolated *V. nigripulchritudo* strains (TUMSAT- V. nig1, TUMSAT- V. nig2 and TUMSAT- V. nig3) were separately cultured in 3 mL of HI broth in 15 mL falcon tubes. The inoculated samples were incubated for 24 hours at 25 $^{o}$C in shaker (BIO shaker -BR 40LF). 1 mL of the cultured bacterial broth was centrifuged at 50,000 rpm for 10 min and the bacterial pellet was collected for the extraction of bacterial DNA.

The bacterial cell disruption was carried out by the resuspension of the obtained bacterial pellet in 5 mL of Buffer G3 which contains RNase. The samples were treated with 25 µL of proteinase- K and incubated the mixture at 37 °C for 40 min. After the incubation and mixing by vortex, 1.2mL of Buffer G4 was added and again incubated at 60 °C for 1 hour to obtain a clear lysate of the sample.

AXG 100 column was used to bind the extracted DNA from the isolated bacteria by the equilibration of the column with Buffer N2. 5 mL of Buffer N2 was added to the sample and vortexed for 15 sec at the maximum speed. After mixing it well, the samples were loaded onto the column allowing it to enter the resin by gravity flow. After the extracted DNA bound, the AXG 100 column was washed with 4 mL of Buffer N3 for 3 times. After changing the washed column with bound DNA into a new falcon tube, 4mL of Buffer N5 was added to elute the bound DNA. After the elution step, 3.5 mL of ice-cold isopropanol was added to precipitate the

DNA. After incubating the samples at room temperature for 30 min, the samples were centrifuged for 15000 rpm for 25 min at 4 °C to obtain the precipitated DNA.

After getting the precipitated DNA pellet, it was re dissolved in 30 µL of TE buffer. After keeping the samples in overnight, all the liquid was collected to 1.5mL Eppendorf tubes. After detecting the integrity of DNA by Agarose gel electrophoresis and measuring the quantity by Qubit fluorometer. The extracted DNA samples were stored at -30 °C until further use for analysis.

### 3.3.2 Illumina sequencing

Illumina sequencing was conducted using the extracted DNA samples of the isolated strains of *V. nigripulchritudo* (TUMSAT- V. nig1, TUMSAT- V. nig2 and TUMSAT- V. nig3) to get a better insight about their genomic features.

**Library preparation**

10 µL of Tagment DNA buffer (TD) and 5 µL of 1 ng extracted DNA form each isolated samples were added into PCR tubes. After mixing by pipetting, 5µL of amplicon Tagment mix (ATM) was added to each tube. After mixing by pipetting, the samples were centrifuged at 30,000 rpm at 20 °C for 1 min. After placing the tubes on thermal cycler, TAG program was run to get the tagmented DNA with 55 °C for 5 min in MiniAmp thermal cycler (Applied Bio Systems, USA).

5 µL of the index adapters of i7 and i5 were added to each samples according to the index adapter kit type without overlapping with each other. 15 µL of Nextera PCR master mix (NPM) was added to each well. After mixing by pipetting, the samples were centrifuged at 28000 rpm at 20 °C for 1 minute. The samples were placed for running the NXT PCR program with amplification conditions were 3 min at 72 °C and 30 sec for 95 °C followed by 12 cycles of 95 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 30 sec, and a final extension step of 72 °C for 5 min.

**Clean up of libraries**

50 µL of the supernatant was transferred from each sample to the corresponding new tube set and 90 µL of Illumina purification beads (IPB) were added to each well containing the supernatant. After sealing the tubes, those were mixed by pipetting and incubate at room temperature for 5 min. The samples were placed on a magnetic stand and wait until the liquid gets clear. Without disturbing the beads, all the supernatant was removed and discarded. After

washing the beads with fresh 80 % EtOH while the tubes were remaining in the magnetic stand, the samples were incubated for 30 sec at room temperature. Without disturbing the beads, all the supernatant was discarded.

After air drying of the samples on the magnetic stand, the tubes were taken out. 40 µL of resuspension buffer (RSB) was added and mixed by pipetting. After incubating for 2 min at room temperature, again the tubes were placed on the magnetic stand and kept until the lysate gets clear. Then, 30 µL of the supernatant from each sample was collected to new tubes.

**Check the library quality**

The quality and the quantity of the obtained DNA libraries were checked by using Agilent Technology 2100 Bioanalyzer using high sensitivity DNA kit and qubit fluorometer. After checking the quality and the quantity of the extracted DNA, the dilution of the samples was performed to get 10 nmol/L libraries with the addition of required amounts of the RSB.

**Pooling and sequencing using Illumina Miseq**

10 µL of each diluted DNA libraries (10 nmol/L) were pooled together in 1.5 mL Eppendorf tube and mixed well by pipetting. Then the pooled library of extracted DNA with 10 nmol/L was diluted and prepared 4 nmol/L sample with the addition of RSB. Then, 5 µL of 4 nmol/L DNA libraries were denatured with 5 µL of 0.02 mol/g NaOH to obtain 2 nmol/L DNA samples to be loaded for the Illumina Miseq to get the sequence data of the extracted DNA of isolated *V. nigripulchritudo* (TUMSAT- V. nig1, TUMSAT- V. nig2 and TUMSAT- V. nig3).

### 3.3.3 Nanopore sequencing

**Library preparation**

The extracted DNA from the isolated strains of *V. nigripulchritudo* (TUMSAT- V. nig1, TUMSAT- V. nig2 and TUMSAT- V. nig3) were sequenced using rapid sequencing gDNA - barcoding with nanopore GridION platform. 7.5 µL of 400 ng of genomic DNA was mixed with 2.5 µL of fragmentation mix RBQ 1-12 for the respective DNA samples. After mixing, the samples were incubated at 30 °C for 1 minute and then 80 °C for 1 minute. After cooling the barcoded samples, those were pooled in a 1.5 mL Eppendorf DNA LoBind tube.

The pooled barcoded DNA sample was resuspended with AMPure XP beads with an equal volume as the pooled sample volume by vortex. The sample was incubated on a Hula mixer for 5 min at room temperature. After spinning down of the sample, it was placed on a magnetic stand and the supernatant was removed carefully. While keeping the sample tube on magnetic

stand, the beads were washed two times with 200 µL of freshly prepared 70 % ETOH without disturbing the pellet. After the complete removal of EtOH, it was allowed to dry. 10 µL of elution buffer was added and incubated for 2 min at room temperature. While keeping on the magnetic stand until gets the clear and colorless elute, the 10 µL of the eluted DNA was transferred into a new 1.5 mL Eppendorf DNA LoBind tube. The eluted DNA was added with 1 µL of RAP and mixed gently. The sample was incubated for 5 min at room temperature.

**Priming and loading the SpotON flow cell**

In the nanopore sequencing machine, 800 µL of the priming mix was loaded into the flow cell via the priming port and allow to move the priming mix for 5 min. Then, 200 µL of the priming mix was loaded to flow cell via the priming port. After mixing well of the prepared DNA library by pipetting, 75 µL of the sample was loaded to the flow cell via SpotON sample port and allowed to be sequenced using nanopore GridION platform. After sequencing, the fast5files were base called using Guppy v.4.0.1 with the settings configdna_r9.4.1_450bps_hac and qscore_ filtering.

### 3.3.4 Construction of genome assembly

The Illumina short reads with Nanopore long reads were assembled to produce hybrid complete genome assemblies of *V. nigripulchritudo* genomes. The raw Illumina sequencing data were quality assessed using fastp v.0.20.2 (Chen *et al*., 2018) with default settings. The Nanopore reads were de novo assembled using Flye v.2.7 (Kolmogorov *et al*., 2019) with the settings nano-raw and genome-size 7M.

After getting the nanopore and Illumina assemblies, the circular topology of the chromosomes and plasmids was confirmed using Bandage (Wick *et al*., 2015). Also, Integrated Genomic Viewer was used to check whether the Illumina and nanopore assemblies are aligned and correctly mapped.

The assemblies got from the Illumina reads and Nanopore reads were aligned using minimap2 v.2.17 and samtools 1.15.1 (Li, 2018). The resulting BAM files were used to improve the accuracy of assemblies by HyPo v.1.0.2 with the settings s 6 m and c 50 (Kundu *et al*., 2018). The assembly quality was checked using Quast v.5.0.2 with default options while Benchmarking Universal Single-Copy Orthologs (BUSCO) v.4.1.was used for assessing the assembly completeness.

### 3.3.5 Genome annotation

The obtained hybrid genome assemblies of the isolated strains of *V. nigripulchritudo* (TUMSAT- V. nig1, TUMSAT- V. nig2 and TUMSAT- V. nig3) were annotated for retrieving the information encoded within different sequence patterns to identify the location and function of those genes. Rapid Annotations using Subsystems Technology (RAST) server v.2.0 (http://rast.nmpdr.org/) (Brettin *et al*., 2015) and Prokka v.1.13.3 (Seemann, 2014) with the settings force, rfam, kingdom bacteria, gram neg, genus Vibrio, use genus for predicting the protein-coding sequences (CDSs), components of RNA; tRNAs, rRNAs, tmRNA and misc RNA found in the genomes. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to determine the gene distribution of the genomes. BlastKOALA was used to assign K numbers to the obtained sequence data by BLAST searches. Visualization of the genomic features were performed by CGView (Stothard and Wishart, 2005).

For the prediction of virulence factors, the related genes and their locations were identified using virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) (Liu *et al*., 2019). The presence of the antimicrobial resistance (AMR) gene family and resistance mechanisms were identified by the Resistance Gene Identifier (RGI) (https://card.mcmaster.ca/analyze/rgi) which is based on the comprehensive antibiotic resistance database (CARD) (Alcock *et al*., 2020).

### 3.3.6 Presence of mobile genetic elements

For the identification the presence of genomic islands in the respective genomes, IslandViewer4 (http://www.pathogenomics.sfu.ca) was used (Bertelli *et al*., 2017) while PHAge Search Tool Enhanced Release (PHASTER) (https://phaster.ca/) was performed to detect the presence of prophages in the genomes of the isolated strains of *V. nigripulchritudo* ( Arndt *et al*., 2016).

### 3.3.7 Comparative whole genome analysis

#### 3.3.7.1 Prediction of clusters of orthologous groups (COGs)

Orthologous genes usually retain similar functions as those of their ancestral genes during evolution. For the identification of the clusters of genes which are evolved from a common ancestral gene, OrthoVenn 2 web tool (https://orthovenn2.bioinfotoolkits.net/task/create) with default parameters E-value 1e-2 and inflation value 1.5 (Xu *et al*., 2019) was used.

The genomes of the three isolated strains of *V. nigripulchritudo* (TUMSAT- V. nig1, TUMSAT- V. nig2 and TUMSAT- V. nig3) and 2 genomes of reference strains of *V. nigripulchritudo*

(TUMSAT-TG-2018 isolated from Japan and SFn1-2013 isolated from New Caledonia) retrieved from the NCBI database were used for the prediction of the orthologous genes in the respective genomes.

#### 3.3.7.2 Phylogenetic tree reconstruction

For the whole genome comparison, a phylogenetic analysis was conducted based on whole-genome proteome data of *V. nigripulchritudo* genomes using Type Strain Genome Server (https://tygs.dsmz.de) with iTOL to describe the genetic relatedness among *V. nigripulchritudo* strains (Henz *et al*., 2005).

The genomes of the three isolated strains of *V. nigripulchritudo* (TUMSAT- V. nig1, TUMSAT-V. nig2 and TUMSAT- V. nig3) and 2 genomes of reference strains of *V. nigripulchritudo* (TUMSAT-TG-2018 isolated from Japan and SFn1-2013 isolated from New Caledonia) retrieved from the NCBI database were used for the construction of the phylogenetic tree. The phylogenetic tree was built using FastME v.2.1.6.1 from whole and visualized by Interactive Tree of Life (iTOL) v.5 (https://itol.embl.de/) (Letunic and Bork, 2021).

## 3.4 Results

### 3.4.1 Genome assembly

In the present study, the Illumina and Nanopore sequencing platforms for yielding chromosome level hybrid assemblies were used to investigate the genomic features of three strains of *V. nigripulchritudo* to get a better insight about their genomic features. In each strain, it has been found with the presence of two circular DNA chromosomes which is consistent with the results from other *Vibrio* species. The sizes of both chromosomes were relatively constant among the isolated strains of *V. nigripulchritudo* genomes. Chromosome I size is ranged from 4.02 to 4.07 Mb, the GC content in average 46 % and the number of predicted CDSs ranged from 3,563 to 3,644 (Figure 3.1a). Chromosome II sizes ranged from 2.16 to 2.18 Mb, with an average GC content of 45.5 % and the number of predicted CDSs ranged from 1,970 to 1,987 for all the three strains of *V. nigripulchritudo* (Figure 3.1b). Only the TUMSAT-V. nig1 strain is with 02 plasmids having 351,696 bp and 172,733 bp respectively (Figure 3.2a and b). The other two strains of TUMSAT-V. nig2 and TUMSAT-V. nig3 are occupied with one plasmid having 187,362 bp and 199,257 bp respectively (Table 3.1). BUSCO analysis yielded 99.8 % of BUSCO completeness, indicating our genome assemblies were of high quality.

Table 3.1 Genome assembly statistics and annotation information of the isolated *V. nigripulchritudo* strains

| Bacterial strain | Total length (bp) | % GC content | #of CDS | r RNA | t RNA | tm RNA | misc RNA |
|---|---|---|---|---|---|---|---|
| **TUMSAT-V. nig 1** | **6757626** | **45.7** | **6157** | **31** | **110** | **1** | **56** |
| Chromosome I | 4072596 | 46.1 | 3644 | 31 | 104 | 1 | 45 |
| Chromosome II | 2160601 | 45.5 | 1971 | 0 | 6 | 0 | 9 |
| Plasmid 1 | 351696 | 44 | 354 | 0 | 0 | 0 | 2 |
| Plasmid 2 | 172733 | 44.6 | 203 | 0 | 0 | 0 | 0 |
| **TUMSAT-V.nig 2** | **6389377** | **45.9** | **5689** | **31** | **109** | **1** | **54** |
| Chromosome I | 4020340 | 46 | 3583 | 31 | 103 | 1 | 44 |
| Chromosome II | 2181675 | 45.7 | 1970 | 0 | 6 | 0 | 10 |
| Plasmid 1 | 187362 | 46.3 | 162 | 0 | 0 | 0 | 0 |
| **TUMSAT-V. nig 3** | **6441463** | **45.9** | **5778** | **31** | **109** | **1** | **54** |
| Chromosome I | 4052685 | 46 | 3610 | 31 | 103 | 1 | 44 |
| Chromosome II | 2189521 | 45.7 | 1987 | 0 | 6 | 0 | 10 |
| Plasmid 1 | 199257 | 46.5 | 184 | 0 | 0 | 0 | 0 |

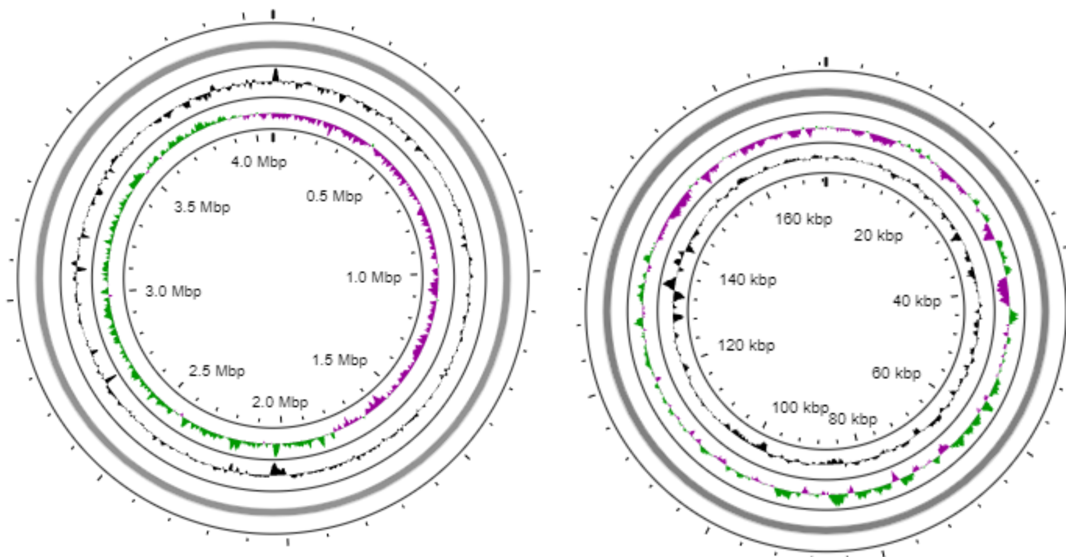


Figure 3.1: Circular genome map of *V. nigripulchritudo* -TUMSAT-V. nig1 (A) chromosome I & (B) chromosome II

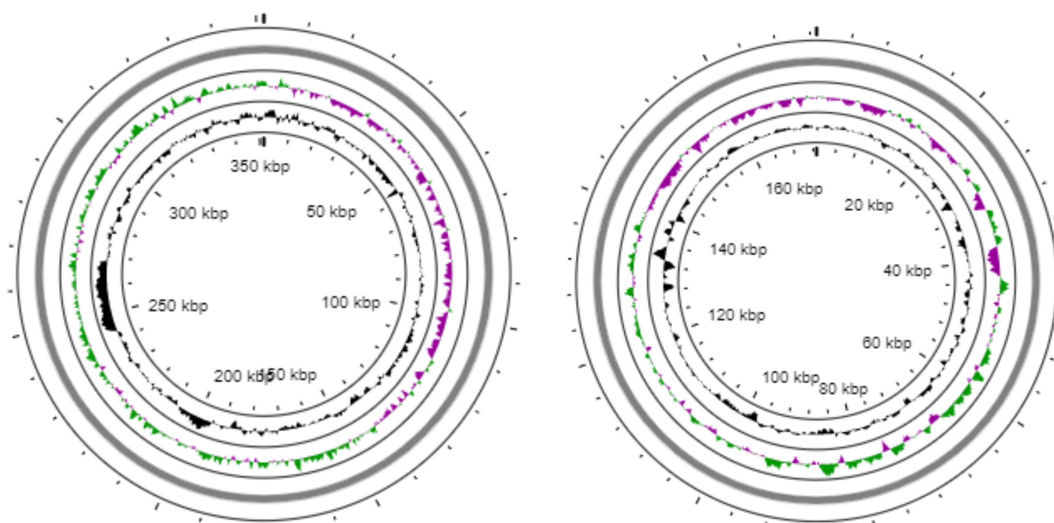


Figure 3.2 : Circular genome map of *V. nigripulchritudo* -TUMSAT-V.nig1 (A) plasmid 1 & (B) plasmid 2

Subsystem information generated by RAST for *V. nigripulchritudo* TUMSAT-V. nig1 showed that all the virulence and disease related subsystems and the phages and prophages are in chromosome I and chromosome II. Among the total of 56 feature counts in virulence, disease and defense subsystem, the chromosome I consists with 33 feature counts followed by 20 in chromosome II. There were no virulence related subsystems found in plasmids (Figure 3.3 a, b,c, ). Instead of that, the plasmid contains the genes involved in their metabolism like membrane transportation, regulation and cell signaling, and metabolism.

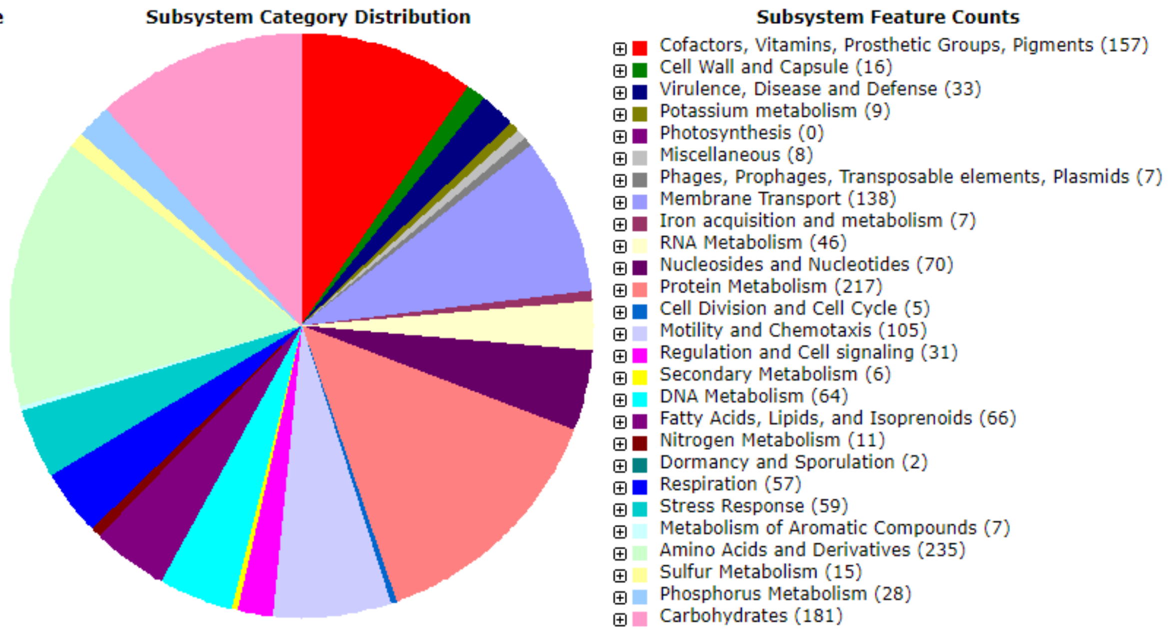


Figure 3.3 (a): Subsystem information generated by RAST for *V. nigripulchritudo* TUMSAT-V. nig1- Chromosome I

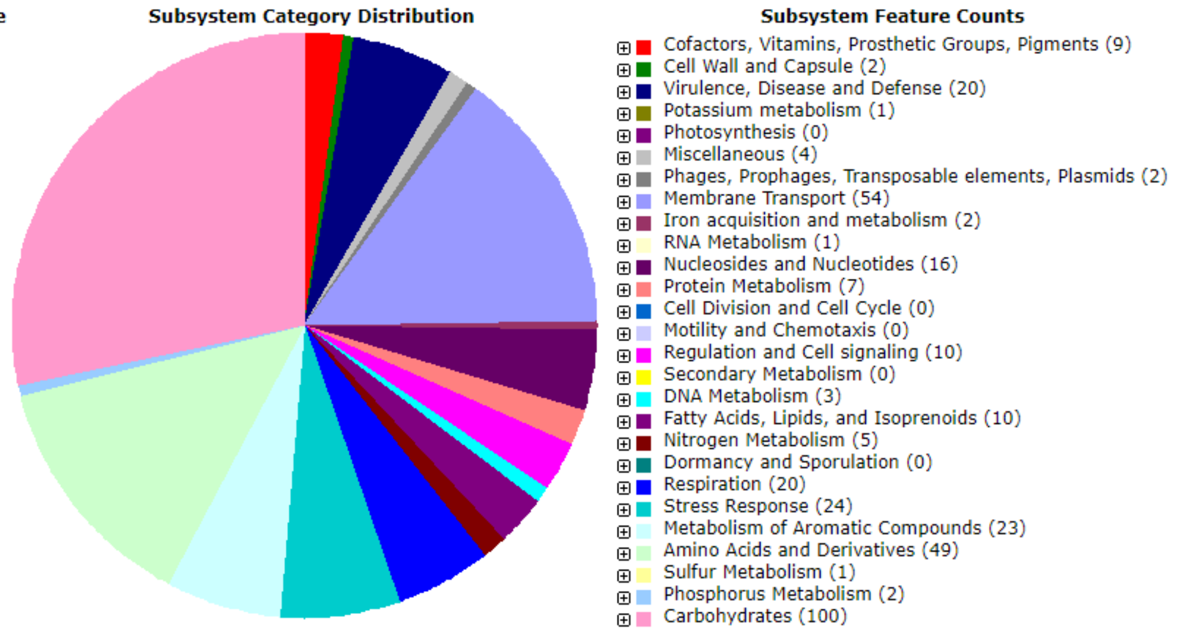


Figure 3.3 (b): Subsystem information generated by RAST for *V. nigripulchritudo* TUMSAT-V. nig1- Chromosome II

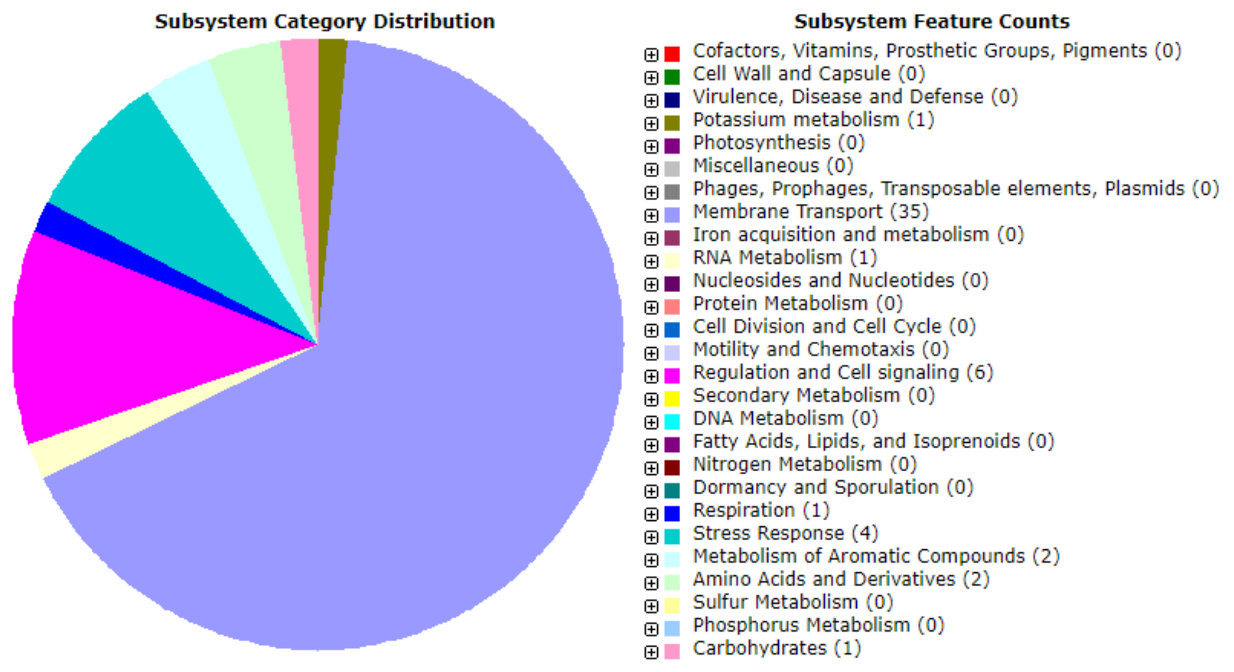


Figure 3.3 (c): Subsystem information generated by RAST for *V. nigripulchritudo* TUMSAT-V. nig1- plasmid I

The gene distribution of the isolated bacterial strains of *V. nigripulchritudo* was analyzed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) classification. KEGG is a database resource for understanding high-level functions and utilities of the biological system. In here, BlastKOALA was used to assign K numbers to the obtained sequence data by BLAST searches. So, it provides a summarized way of how the genes are distributed in the isolated stains with the number of genes for specific functional category. In these isolates, the highest number of genes are involved in environmental information processing while the least number for bio synthesis for other secondary metabolites (Figure 3.4).

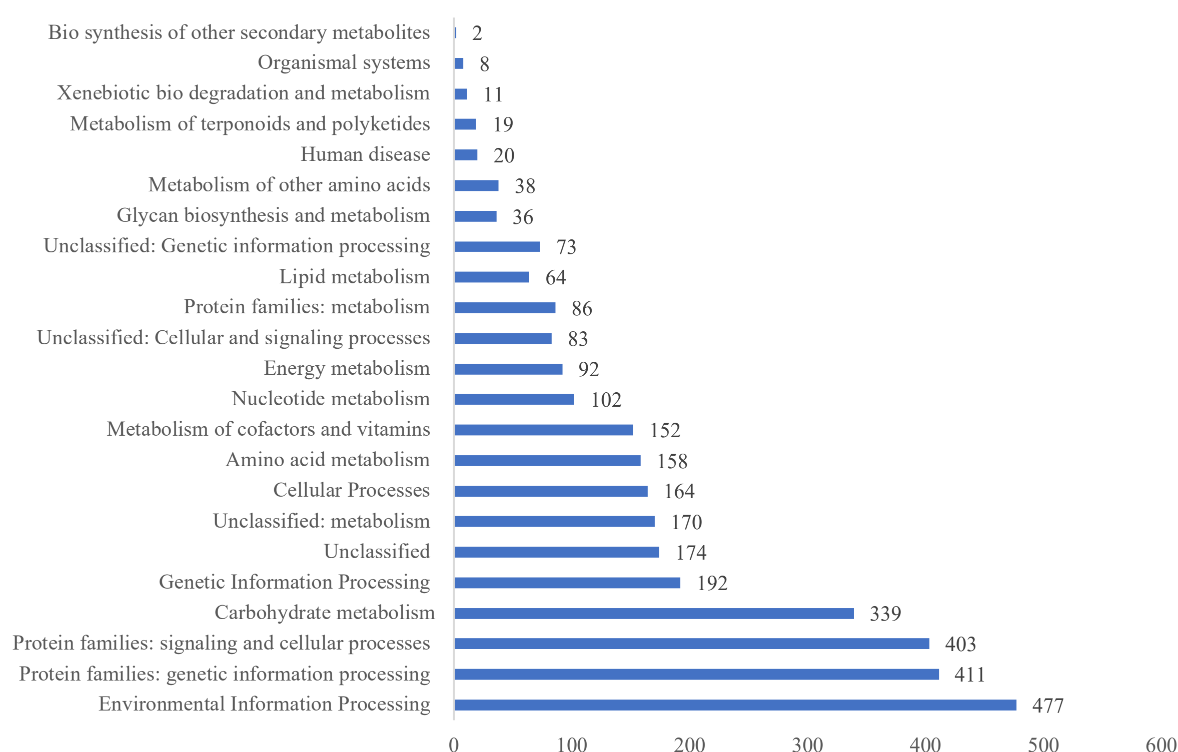


Figure 3.4: Gene distribution based on KEGG classification of *V. nigripulchritudo* TUMSAT-V. nig1

### 3.4.2 Identification of virulence related genes

Virulence Factor Database (VFDB) revealed several putative virulence factors found in the genomes of *V. nigripulchritudo* including genes involved in adherence, anti-phagocytosis, chemotaxis and motility, iron uptake and acquisition, quorum sensing, secretion systems and toxins. All the virulence related genes are in either chromosome I or chromosome II in all the genomes of isolated strains of *V. nigripulchritudo* indicating the involvement of the chromosomes for the virulence of these bacteria (Table 3.2).

In the chromosome I of the isolated stains of *V. nigripulchritudo*, five major virulence related traits are found including, Adherence, Antiphagocytosis, Chemotaxis and motility, Quorum sensing and Secretion system while the chromosome II is having another two more virulence factors including iron uptake and toxins instead of those found in chromosome I.

Adherence is an essential step in bacterial pathogenesis or infection, required for colonizing a new host. Adhesion occurs when adhesive molecules expressed on the bacterial surface bind to host surface receptors. So, in here, Mannose-sensitive hemagglutinin and Type IV pilus are identified in chromosome I while Accessory colonization factor is found in chromosome II. For impeding or preventing the action of phagocytes or the occurrence of phagocytosis, the presence

of capsular polysaccharides in both chromosome I and II is used as a virulence factor by *V. nigripulchritudo*.

Quorum-sensing is an intraspecies interaction mechanism characteristic of bacteria and fungi which allows individual bacteria within colonies to coordinate and carry out colony-wide functions such as: sporulation, bioluminescence, virulence, conjugation, competence, and biofilm formation (Turovskiy *et al*., 2007). In the genomes of isolated strains of *V. nigripulchritudo*, Auto inducer-2 in chromosome I and Cholerae auto inducer_1 in chromosome II is identified to be used for quorum sensing.

Flagella constitute one of the most effective bacterial antigens and the immune system directs its counterattacks against it and found in both chromosomes of the isolated *V. nigripulchritudo* strains. Chemotaxis also occurs in bacteria within the rhizosphere and in aquatic environment. Bacterial secretion systems are protein complexes present on the cell membranes of bacteria for secretion of substances. Specifically, they are the cellular devices used by pathogenic bacteria to secrete their virulence factors (mainly of proteins) to invade the host cells. In here, it has found with EPS type II secretion system in chromosome I and VAS effector proteins with VAS type VI secretion system in chromosome II of the isolated strains.

Among the virulence factors found in the isolated strains, related genes for iron uptake and toxins are only found in chromosome II. For the iron uptake, it has been found that *V. nigripulchritudo* has Vibriobactin, siderophore that helps the microbial system to acquire iron, Enterobactin receptors and Heme receptors. Hemolysin is a potential virulence factor produced by most of the *Vibrio* sp. which is considered as an exotoxin from bacteria which causes lysis of red blood cells. In *Vibrio nigripulchritudo*, it has been found as one of the major virulence factors located in chromosome II with *tlh* gene.

Table 3.2 Potential virulence factor profiles of the three strains of *V. nigripulchritudo* predicted using the virulence factor database (VFDB)

| Classification | Virulence factor | Related gene | Location |
|---|---|---|---|
| Adherence | Accessory colonization factor | <u>*acfB*</u> | Chromosome II |
| | Mannose-sensitive hemagglutinin (MSHA type IV pilus) | *mshA* | Chromosome I |
| | Type IV pilus | *pilA, pilB, pilC, pilD* | Chromosome I |
| Antiphagocytosis | Capsular polysaccharide | *wbfB, wbfV/wcvB, wbfY* | Chromosome I |
| | | *cpsA* | Chromosome II |
| Chemotaxis and motility | Flagella | *cheB,cheR,cheV,cheW, cheY, cheZ, filM, flaA, flaC, flaD,flaE, flgA, flgB, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, flgL, flgM, flgN, flhA, flhB, flhF, flhG, fliA, fliD, fliE, fliF, fliG, fliH, fliI, fliJ, fliL, fliN, fliO, flip, fliQ, fliR, fliS, flrA, flrB, flrC, motA, motB, motX, motY* | Chromosome I |
| | | *cheA* | Chromosome II |
| Iron uptake | Enterobactin receptors | *vctA* | Chromosome II |
| | Heme receptors | *hutA* | Chromosome II |
| | Periplasmic binding protein dependent ABC transport systems | *vctC, vctD, vctG, viuC, viuD, viuG, viuP* | Chromosome II |
| | Vibriobactin | *vibA, vibB, vibC, vibE, vibF, viuA, viuB* | Chromosome II |

| | | | |
|---|---|---|---|
| Quorum sensing | Auto inducer-2 | *luxS* | Chromosome I |
| | Cholerae auto inducer_1 | *cqsA* | Chromosome II |
| Secretion system | EPS type II secretion system | *epsC, epsE, epsF, epsG, epsH, epsI, epsJ, epsK, epsL, epsM, epsN, gspD* | Chromosome I |
| | VAS effector proteins | *Hcp-2, vgrG-2* | Chromosome II |
| | VAS type VI secretion system | *vasA, vasB, vasC, vasD, vasE, vasF, vasG, vasH, vasI, vasJ, vasK* | Chromosome II |
| Toxins | Thermolabile hemolysin | *Tlh* | Chromosome II |

### 3.4.3 Identification of potential mobile genetic elements

#### 3.4.3.1 Antimicrobial resistant genes

Antimicrobial resistance happens when some of the bacterial cells express with anti-microbial resistance genes (AMR) in their genome. So that, they can develop the ability to defeat the drugs designed to kill them and the resistant bacteria continue to grow causing severe environmental threat with the spreading of AMR genes.

After annotating with the Comprehensive Antibiotic Resistance Database with resistance gene identifier, it has been found 04 major categories of global regulator that represses MdtEF multidrug efflux pump expression (CRP), membrane fusion protein of the multidrug efflux complex AdeFGH (adeF), *Escherichia coli* ParE conferring resistance to fluroquinolone, and *Haemophilus influenzae* PBP3 conferring resistance to beta-lactam (Table 3.3).

Several drugs belonged to these are found creating a resistance to these antibiotics by the isolated strains. Also, three AMR gene families were found as Resistance nodulation cell division (RND) antibiotic efflux pump (macrolide, fluoroquinolone, tetracycline), Penicillin-binding protein mutations conferring resistance to beta-lactam antibiotics (cephalosporin, cephamycin, penam), and fluoroquinolone resistant parE (fluoroquinolone) in the isolated strains of *V. nigripulchritudo* genomes (Figure 3.5). Two resistant mechanisms for the antibiotic resistivity have been found as antibiotic efflux pump and antibiotic target alteration also.

Table 3.3: The presence of antibiotic resistance genes predicted by Comprehensive Antibiotic Resistance Database (CARD) with resistance gene identifier (RGI)

| RGI criteria | ARO term | AMR gene family | Drug class | Resistant mechanism |
|---|---|---|---|---|
| Strict | CRP (Global regulator that repress MdtEF multidrug efflux pump) | Resistance nodulation cell division (RND) antibiotic efflux pump | Macrolide Fluroquinolone Penam | antibiotic efflux pump |
| Strict | adeF (Membrane fusion protein of multidrug efflux AdeFGH) | Resistance nodulation cell division (RND) antibiotic efflux pump | Fluroquinolone Tetracycline | antibiotic efflux pump |
| Strict | *Escherichia coli* ParE conferring resistance to fluroquinolone | Fluroquinolone resistance ParE | Fluroquinolone | Antibiotic target alteration |
| Strict | *Haemophilus influenzae* PBP3 conferring resistance to beta-lactem | Penicillin binding protein mutation conferring resistance to beta-lactem | Cephalosporin, Cephamycin, Penam | Antibiotic target alteration |

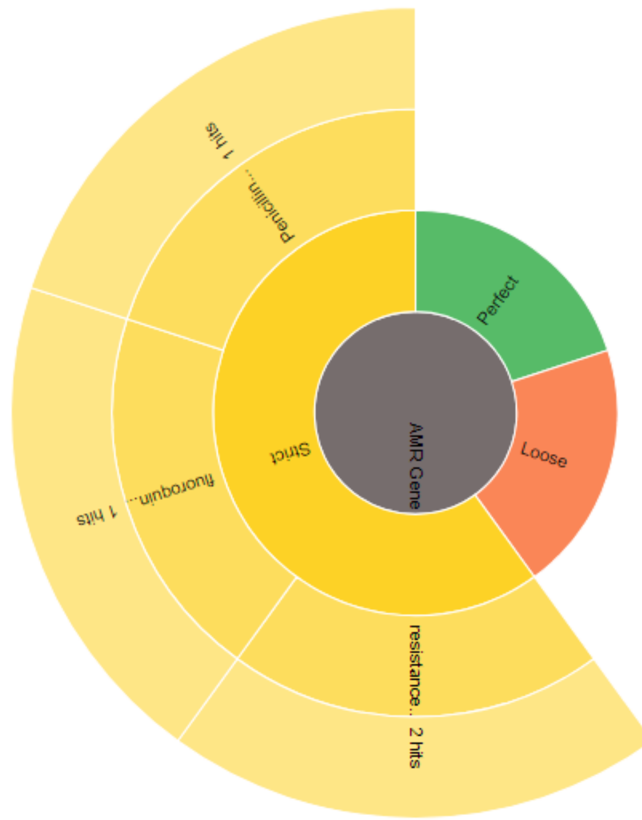


Figure 3.5: Antimicrobial resistance generated by RGI for *V. nigripulchritudo* TUMSAT-V. nig1; resistance-nodulation-cell division (RND) antibiotic efflux pump, fluoroquinolone resistant parE, and Penicillin-binding protein mutations conferring resistance to beta-lactam antibiotics

#### 3.4.3.2 Genomic islands

Genomic islands (GI) are typically recognized as large segments of genomic DNA that range in size from 10 to 200 kb. It is a part of a genome that has evidence of horizontal origins. The presence of GIs in the genomes of the isolated *V. nigripulchritudo* was performed using IslandViewer4. In total, 46 GIs were detected in the *V. nigripulchritudo* TUMSAT-V. nig1 strain while 30 and 36 GIs were found in TUMSAT-V. nig2 and TUMSAT-V. nig3. Strain TUMSAT-V. nig1 had the highest number of regions encoding GIs, suggesting that this strain has experienced numerous HGT events mediated by GIs.

The largest genomic island (100698 bp) of the genome of *V. nigripulchritudo*-TUMSAT V. nig 1 is found with DNA primase DnaG, Twitching motility protein PilT, diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s), Error-prone, lesion bypass DNA polymerase V (UmuC), Error-prone repair protein UmuD, ATP synthase gamma chain (EC 3.6.3.14), Putative DNA-binding protein in cluster with Type I restriction-

modification system, Transposase, ATP-dependent DNA helicase RecQ, DNA topoisomerase I (EC 5.99.1.2), TrbA-like protein along with numerous hypothetical proteins. In the smallest genomic island of the same genome is occupied with 4036 bp consisted with Histone acetyltransferase HPA2 and some hypothetical proteins (Table 3.4).

Some virulence genes including T1SS secreted agglutinin RTX, Flagellar protein, Hemolysin-related protein, T6SS PAAR-repeat protein and Toxin HigB / Protein kinase domain of HipA are found in genomic islands of the TUMSAT-V. nig1 creating pathogenic islands. Also, GIs encoding Beta-lactamase class C-like and penicillin binding were identified in the respective genomes (Figure 3.6)

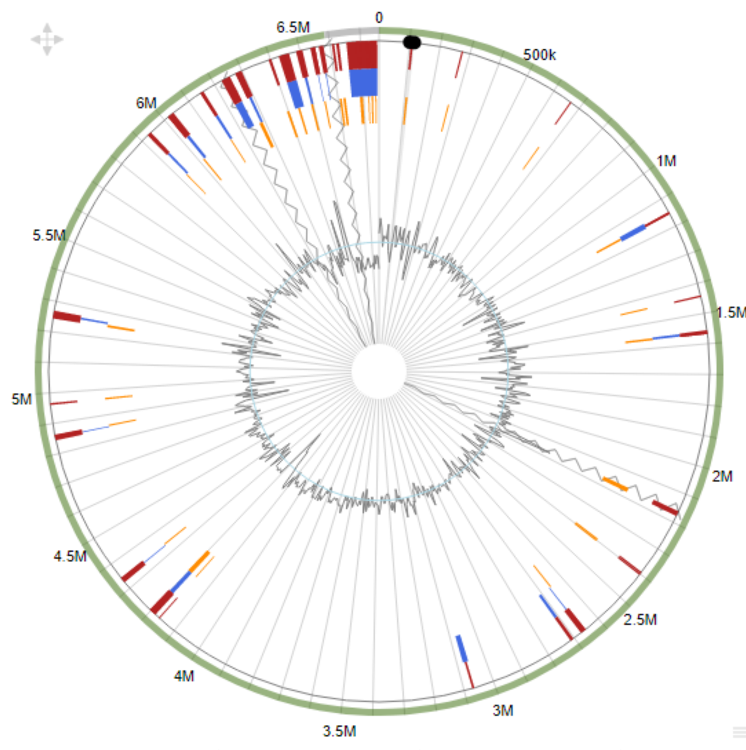


Figure 3.6: The genome of *V. nigripulchritudo* TUMSAT-V. nig1 aligned with the reference genome of *V. nigripulchritudo* SFn1 to indicate the presence of genomic islands

Table 3.4 The genomic islands present in the genome of *V. nigripulchritudo* TUMSAT-V. nig1

| Island start | Island end | Length | Gene start | Gene end | Product |
|---|---|---|---|---|---|
| 103818 | 111269 | 7451 | 103818 | 104174 | hypothetical protein |
| 103818 | 111269 | 7451 | 104187 | 104744 | RNA:NAD 2'-phosphotransferase |
| 103818 | 111269 | 7451 | 105076 | 105276 | hypothetical protein |
| 103818 | 111269 | 7451 | 105515 | 105724 | hypothetical protein |
| 103818 | 111269 | 7451 | 105934 | 107211 | Alkaline serine exoprotease A precursor |
| 103818 | 111269 | 7451 | 107346 | 107702 | hypothetical protein |
| 103818 | 111269 | 7451 | 107715 | 108059 | hypothetical protein |
| 103818 | 111269 | 7451 | 108552 | 108887 | hypothetical protein |
| 103818 | 111269 | 7451 | 109112 | 109450 | hypothetical protein |
| 103818 | 111269 | 7451 | 109511 | 109840 | hypothetical protein |
| 103818 | 111269 | 7451 | 109848 | 110129 | hypothetical protein |
| 103818 | 111269 | 7451 | 110229 | 110573 | hypothetical protein |
| 103818 | 111269 | 7451 | 110577 | 110906 | hypothetical protein |
| 103818 | 111269 | 7451 | 110919 | 111269 | hypothetical protein |
| 271732 | 276848 | 5116 | 271732 | 272508 | hypothetical protein |
| 271732 | 276848 | 5116 | 272477 | 273217 | hypothetical protein |
| 271732 | 276848 | 5116 | 273342 | 274598 | hypothetical protein |
| 271732 | 276848 | 5116 | 274585 | 275160 | hypothetical protein |
| 271732 | 276848 | 5116 | 275694 | 276848 | hypothetical protein |
| 664079 | 668526 | 4447 | 664079 | 665572 | hypothetical protein |
| 664079 | 668526 | 4447 | 665905 | 666600 | hypothetical protein |
| 664079 | 668526 | 4447 | 666940 | 667287 | hypothetical protein |
| 664079 | 668526 | 4447 | 667642 | 668526 | hypothetical protein |
| 1148785 | 1156423 | 7638 | 1148785 | 1148922 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 1148785 | 1156423 | 7638 | 1148975 | 1150060 | Thymidylate synthase (EC 2.1.1.45) |
| 1148785 | 1156423 | 7638 | 1150605 | 1151636 | hypothetical protein |
| 1148785 | 1156423 | 7638 | 1151756 | 1152190 | Mobile element protein |
| 1148785 | 1156423 | 7638 | 1152280 | 1152771 | hypothetical protein |
| 1148785 | 1156423 | 7638 | 1152765 | 1153427 | hypothetical protein |
| 1148785 | 1156423 | 7638 | 1153528 | 1154985 | USG protein |
| 1148785 | 1156423 | 7638 | 1155926 | 1156168 | hypothetical protein |
| 1148785 | 1156423 | 7638 | 1156208 | 1156423 | Uncharacterized protein YpeB |
| 1148975 | 1156168 | 7193 | 1148975 | 1150060 | Thymidylate synthase (EC 2.1.1.45) |
| 1148975 | 1156168 | 7193 | 1150605 | 1151636 | hypothetical protein |
| 1148975 | 1156168 | 7193 | 1151756 | 1152190 | Mobile element protein |
| 1148975 | 1156168 | 7193 | 1152280 | 1152771 | hypothetical protein |
| 1148975 | 1156168 | 7193 | 1152765 | 1153427 | hypothetical protein |
| 1148975 | 1156168 | 7193 | 1153528 | 1154985 | USG protein |
| 1148975 | 1156168 | 7193 | 1155926 | 1156168 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1440091 | 1440249 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1440348 | 1440677 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1440770 | 1441090 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1441255 | 1441851 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1441976 | 1442563 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1442796 | 1443539 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1443705 | 1444463 | putative type II restriction endonuclease |
| 1440091 | 1445870 | 5779 | 1444492 | 1444944 | hypothetical protein |
| 1440091 | 1445870 | 5779 | 1444983 | 1445870 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1555552 | 1556568 | Mobile element protein |

| | | | | | |
|---|---|---|---|---|---|
| 1555552 | 1568146 | 12594 | 1556687 | 1557331 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1557678 | 1558541 | Mobile element protein |
| 1555552 | 1568146 | 12594 | 1559059 | 1560084 | Mobile element protein |
| 1555552 | 1568146 | 12594 | 1560186 | 1561289 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1561771 | 1562238 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1562779 | 1563522 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1564140 | 1564607 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1565026 | 1565871 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1566395 | 1567201 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1567350 | 1567685 | hypothetical protein |
| 1555552 | 1568146 | 12594 | 1567802 | 1568146 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1560186 | 1561289 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1561771 | 1562238 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1562779 | 1563522 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1564140 | 1564607 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1565026 | 1565871 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1566395 | 1567201 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1567350 | 1567685 | hypothetical protein |
| 1560186 | 1568146 | 7960 | 1567802 | 1568146 | hypothetical protein |
| 2160454 | 2177354 | 16900 | 2160454 | 2160600 | diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s) |
| 2160454 | 2177354 | 16900 | 2161604 | 2168590 | T1SS secreted agglutinin RTX |
| 2160454 | 2177354 | 16900 | 2168556 | 2170613 | T1SS secreted agglutinin RTX |
| 2160454 | 2177354 | 16900 | 2170610 | 2174002 | T1SS secreted agglutinin RTX |
| 2160454 | 2177354 | 16900 | 2174013 | 2177354 | T1SS secreted agglutinin RTX |
| 2392322 | 2398207 | 5885 | 2392322 | 2392627 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 2392322 | 2398207 | 5885 | 2393067 | 2393636 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2393681 | 2393836 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2393971 | 2394366 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2394661 | 2394924 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2395068 | 2395463 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2395593 | 2395991 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2396147 | 2396269 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2396367 | 2396558 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2396710 | 2396928 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2396973 | 2397245 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2397308 | 2397781 | hypothetical protein |
| 2392322 | 2398207 | 5885 | 2397902 | 2398207 | hypothetical protein |
| 2398715 | 2404220 | 5505 | 2398715 | 2399443 | hypothetical protein |
| 2398715 | 2404220 | 5505 | 2399585 | 2399926 | hypothetical protein |
| 2398715 | 2404220 | 5505 | 2400067 | 2400573 | hypothetical protein |
| 2398715 | 2404220 | 5505 | 2400970 | 2401155 | hypothetical protein |
| 2398715 | 2404220 | 5505 | 2401304 | 2401633 | hypothetical protein |
| 2398715 | 2404220 | 5505 | 2402037 | 2402465 | Mobile element protein |
| 2398715 | 2404220 | 5505 | 2402469 | 2402993 | Mobile element protein |
| 2398715 | 2404220 | 5505 | 2403028 | 2403618 | rRNA methylase |
| 2398715 | 2404220 | 5505 | 2403756 | 2404220 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2653937 | 2654062 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2654183 | 2656546 | Superfamily I DNA/RNA helicase protein |
| 2653937 | 2672699 | 18762 | 2656713 | 2657576 | Superfamily I DNA helicase |
| 2653937 | 2672699 | 18762 | 2658261 | 2658377 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2658405 | 2658623 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 2653937 | 2672699 | 18762 | 2658688 | 2658963 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2658956 | 2660095 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2660508 | 2660621 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2660661 | 2660789 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2661575 | 2662024 | L-alanine exporter AlaE |
| 2653937 | 2672699 | 18762 | 2662317 | 2664743 | Two-component system sensor histidine kinase |
| 2653937 | 2672699 | 18762 | 2664863 | 2665432 | hypothetical protein |
| 2653937 | 2672699 | 18762 | 2665498 | 2666559 | Quinolinate synthetase (EC 2.5.1.72) |
| 2653937 | 2672699 | 18762 | 2666755 | 2667543 | Cell division coordinator CpoB |
| 2653937 | 2672699 | 18762 | 2667557 | 2668093 | Tol-Pal system peptidoglycan-associated lipoprotein PAL |
| 2653937 | 2672699 | 18762 | 2668163 | 2669515 | Tol-Pal system beta propeller repeat protein TolB |
| 2653937 | 2672699 | 18762 | 2669528 | 2670583 | TolA protein |
| 2653937 | 2672699 | 18762 | 2670602 | 2671042 | Tol biopolymer transport system, TolR protein |
| 2653937 | 2672699 | 18762 | 2671042 | 2671728 | Tol-Pal system protein TolQ |
| 2653937 | 2672699 | 18762 | 2671718 | 2672155 | Tol-Pal system-associated acyl-CoA thioesterase |
| 2653937 | 2672699 | 18762 | 2672291 | 2672599 | Protein YbgE |
| 2653937 | 2672699 | 18762 | 2672589 | 2672699 | Cytochrome d ubiquinol oxidase subunit X (EC 1.10.3.-) |
| 2653937 | 2658963 | 5026 | 2653937 | 2654062 | hypothetical protein |
| 2653937 | 2658963 | 5026 | 2654183 | 2656546 | Superfamily I DNA/RNA helicase protein |
| 2653937 | 2658963 | 5026 | 2656713 | 2657576 | Superfamily I DNA helicase |
| 2653937 | 2658963 | 5026 | 2658261 | 2658377 | hypothetical protein |
| 2653937 | 2658963 | 5026 | 2658405 | 2658623 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 2653937 | 2658963 | 5026 | 2658688 | 2658963 | hypothetical protein |
| 2653937 | 2658963 | 5026 | 2658956 | 2660095 | hypothetical protein |
| 2703574 | 2715053 | 11479 | 2703574 | 2704248 | RecA/RadA recombinase |
| 2703574 | 2715053 | 11479 | 2704250 | 2705650 | hypothetical protein |
| 2703574 | 2715053 | 11479 | 2705659 | 2708730 | Error-prone repair homolog of DNA polymerase III alpha subunit (EC 2.7.7.7) |
| 2703574 | 2715053 | 11479 | 2708778 | 2709422 | hypothetical protein |
| 2703574 | 2715053 | 11479 | 2709483 | 2710409 | hypothetical protein |
| 2703574 | 2715053 | 11479 | 2710925 | 2713000 | ATP-dependent helicase DinG/Rad3 |
| 2703574 | 2715053 | 11479 | 2713039 | 2713818 | FIG011065: hypothetical protein |
| 2703574 | 2715053 | 11479 | 2713815 | 2714366 | Primosomal replication protein N prime prime |
| 2703574 | 2715053 | 11479 | 2714363 | 2714521 | Uncharacterized protein YbaM |
| 2703574 | 2715053 | 11479 | 2714538 | 2715053 | FIG002577: Putative lipoprotein precursor |
| 2703574 | 2715053 | 11479 | 2715050 | 2715616 | FIG026291: Hypothetical periplasmic protein |
| 3065252 | 3072458 | 7206 | 3065252 | 3065887 | Flagellar protein FlgO |
| 3065252 | 3072458 | 7206 | 3066046 | 3067185 | Flagellar protein FlgT |
| 3065252 | 3072458 | 7206 | 3067404 | 3067763 | Phage integrase |
| 3065252 | 3072458 | 7206 | 3067896 | 3069089 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3069111 | 3069644 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3069656 | 3069820 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3069821 | 3070042 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3070079 | 3070216 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3070465 | 3070812 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3070802 | 3071302 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 3065252 | 3072458 | 7206 | 3071487 | 3071846 | hypothetical protein |
| 3065252 | 3072458 | 7206 | 3072021 | 3072458 | Azurin |
| 4161675 | 4165737 | 4062 | 4161675 | 4162406 | Beta-1,4-galactosyltransferase |
| 4161675 | 4165737 | 4062 | 4162496 | 4163170 | Hypohetical Teichoic Acid Biosynthesis Protein |
| 4161675 | 4165737 | 4062 | 4163172 | 4164479 | hypothetical protein |
| 4161675 | 4165737 | 4062 | 4164487 | 4165737 | 3-deoxy-D-manno-octulosonic acid transferase (EC 2.4.99.12)(EC 2.4.99.13) |
| 4161675 | 4165737 | 4062 | 4165734 | 4166768 | ADP-heptose--lipooligosaccharide heptosyltransferase II |
| 4180544 | 4205007 | 24463 | 4180544 | 4181167 | Acetyltransferase (isoleucine patch superfamily) |
| 4180544 | 4205007 | 24463 | 4181168 | 4182241 | putative ketoacyl-ACP synthase |
| 4180544 | 4205007 | 24463 | 4182251 | 4182469 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4182476 | 4183225 | Oxidoreductase, short-chain dehydrogenase/reductase family |
| 4180544 | 4205007 | 24463 | 4183243 | 4183530 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4183537 | 4184238 | Oxidoreductase, short-chain dehydrogenase/reductase family |
| 4180544 | 4205007 | 24463 | 4184235 | 4185587 | Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3) |
| 4180544 | 4205007 | 24463 | 4185609 | 4186778 | UDP-N,N'-diacetylbacillosamine 2-epimerase (hydrolyzing) (EC 3.2.1.184) |
| 4180544 | 4205007 | 24463 | 4186775 | 4187857 | N-acetylneuraminate synthase (EC 2.5.1.56) |
| 4180544 | 4205007 | 24463 | 4187857 | 4188612 | Imidazole glycerol phosphate synthase cyclase subunit |
| 4180544 | 4205007 | 24463 | 4188609 | 4189205 | Imidazole glycerol phosphate synthase amidotransferase subunit (EC 2.4.2.-) |

| | | | | | |
|---|---|---|---|---|---|
| 4180544 | 4205007 | 24463 | 4189219 | 4190433 | Legionaminic acid biosynthesis protein PtmG |
| 4180544 | 4205007 | 24463 | 4190435 | 4191484 | D-glycero-alpha-D-manno-heptose 1-phosphate guanylyltransferase (EC 2.7.7.71) |
| 4180544 | 4205007 | 24463 | 4191481 | 4192197 | N-Acetylneuraminate cytidylyltransferase (EC 2.7.7.43) |
| 4180544 | 4205007 | 24463 | 4192448 | 4193725 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4193725 | 4194555 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4194539 | 4195093 | Lipid carrier : UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-) |
| 4180544 | 4205007 | 24463 | 4195096 | 4196874 | Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4) |
| 4180544 | 4205007 | 24463 | 4196906 | 4198102 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4198537 | 4198746 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4200027 | 4200167 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4200198 | 4200314 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4200367 | 4202274 | Nucleoside-diphosphate sugar epimerase/dehydratase |
| 4180544 | 4205007 | 24463 | 4202393 | 4203379 | hypothetical protein |
| 4180544 | 4205007 | 24463 | 4203497 | 4203622 | Mobile element protein |
| 4180544 | 4205007 | 24463 | 4204145 | 4204681 | Mobile element protein |
| 4180544 | 4205007 | 24463 | 4204867 | 4205007 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4185609 | 4186778 | UDP-N,N'-diacetylbacillosamine 2-epimerase (hydrolyzing) (EC 3.2.1.184) |
| 4185609 | 4203622 | 18013 | 4186775 | 4187857 | N-acetylneuraminate synthase (EC 2.5.1.56) |
| 4185609 | 4203622 | 18013 | 4187857 | 4188612 | Imidazole glycerol phosphate synthase cyclase subunit |

| | | | | | |
|---|---|---|---|---|---|
| 4185609 | 4203622 | 18013 | 4188609 | 4189205 | Imidazole glycerol phosphate synthase amidotransferase subunit (EC 2.4.2.-) |
| 4185609 | 4203622 | 18013 | 4189219 | 4190433 | Legionaminic acid biosynthesis protein PtmG |
| 4185609 | 4203622 | 18013 | 4190435 | 4191484 | D-glycero-alpha-D-manno-heptose 1-phosphate guanylyltransferase (EC 2.7.7.71) |
| 4185609 | 4203622 | 18013 | 4191481 | 4192197 | N-Acetylneuraminate cytidylyltransferase (EC 2.7.7.43) |
| 4185609 | 4203622 | 18013 | 4192448 | 4193725 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4193725 | 4194555 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4194539 | 4195093 | Lipid carrier : UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-) |
| 4185609 | 4203622 | 18013 | 4195096 | 4196874 | Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4) |
| 4185609 | 4203622 | 18013 | 4196906 | 4198102 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4198537 | 4198746 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4200027 | 4200167 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4200198 | 4200314 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4200367 | 4202274 | Nucleoside-diphosphate sugar epimerase/dehydratase |
| 4185609 | 4203622 | 18013 | 4202393 | 4203379 | hypothetical protein |
| 4185609 | 4203622 | 18013 | 4203497 | 4203622 | Mobile element protein |
| 4326019 | 4344565 | 18546 | 4326019 | 4326300 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4326343 | 4327056 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4327120 | 4327977 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4328200 | 4329567 | IncF plasmid conjugative transfer pilus assembly protein TraH |
| 4326019 | 4344565 | 18546 | 4329568 | 4333023 | IncF plasmid conjugative transfer protein TraG |

| | | | | | |
|---|---|---|---|---|---|
| 4326019 | 4344565 | 18546 | 4333017 | 4333301 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4333385 | 4333726 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4334034 | 4334198 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4334216 | 4334836 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4334826 | 4335383 | Transcriptional activator |
| 4326019 | 4344565 | 18546 | 4335474 | 4338254 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4338314 | 4338427 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4338879 | 4339430 | Phage integrase, site-specific serine recombinase |
| 4326019 | 4344565 | 18546 | 4339473 | 4340294 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4340291 | 4340518 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4340509 | 4342227 | hypothetical protein |
| 4326019 | 4344565 | 18546 | 4342330 | 4343424 | Phage integrase |
| 4326019 | 4344565 | 18546 | 4343522 | 4344565 | tRNA-dihydrouridine(20/20a) synthase (EC 1.3.1.91) |
| 4338314 | 4343424 | 5110 | 4338314 | 4338427 | hypothetical protein |
| 4338314 | 4343424 | 5110 | 4338879 | 4339430 | Phage integrase, site-specific serine recombinase |
| 4338314 | 4343424 | 5110 | 4339473 | 4340294 | hypothetical protein |
| 4338314 | 4343424 | 5110 | 4340291 | 4340518 | hypothetical protein |
| 4338314 | 4343424 | 5110 | 4340509 | 4342227 | hypothetical protein |
| 4338314 | 4343424 | 5110 | 4342330 | 4343424 | Phage integrase |
| 4844334 | 4862958 | 18624 | 4844334 | 4845812 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4846179 | 4847693 | Hemolysin-related protein RbmC |
| 4844334 | 4862958 | 18624 | 4848025 | 4849635 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4850079 | 4850963 | FIG068086: hypothetical protein |
| 4844334 | 4862958 | 18624 | 4851161 | 4853248 | Translation elongation factor G paralog VC2342 |

| | | | | | |
|---|---|---|---|---|---|
| 4844334 | 4862958 | 18624 | 4853328 | 4853861 | Dihydrofolate reductase homolog |
| 4844334 | 4862958 | 18624 | 4853961 | 4854500 | Transcriptional regulator, AcrR family |
| 4844334 | 4862958 | 18624 | 4854516 | 4855472 | Transcriptional regulator, LysR family |
| 4844334 | 4862958 | 18624 | 4855573 | 4855779 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4856419 | 4856862 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4856886 | 4857437 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4857412 | 4858302 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4858292 | 4858681 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4858683 | 4859129 | hypothetical protein |
| 4844334 | 4862958 | 18624 | 4859151 | 4860431 | Beta-lactamase class C-like and penicillin binding proteins (PBPs) superfamily |
| 4844334 | 4862958 | 18624 | 4860685 | 4861329 | Mobile element protein |
| 4844334 | 4862958 | 18624 | 4861759 | 4862655 | Transcriptional regulator, LysR family |
| 4844334 | 4862958 | 18624 | 4862767 | 4862958 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4855573 | 4855779 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4856419 | 4856862 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4856886 | 4857437 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4857412 | 4858302 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4858292 | 4858681 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4858683 | 4859129 | hypothetical protein |
| 4855573 | 4861329 | 5756 | 4859151 | 4860431 | Beta-lactamase class C-like and penicillin binding proteins (PBPs) superfamily |
| 4855573 | 4861329 | 5756 | 4860685 | 4861329 | Mobile element protein |
| 4960567 | 4966917 | 6350 | 4960567 | 4961091 | T6SS PAAR-repeat protein |
| 4960567 | 4966917 | 6350 | 4961091 | 4961615 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 4960567 | 4966917 | 6350 | 4961612 | 4965172 | hypothetical protein |
| 4960567 | 4966917 | 6350 | 4965156 | 4966154 | hypothetical protein |
| 4960567 | 4966917 | 6350 | 4966216 | 4966917 | Lipoate-protein ligase A |
| 5243502 | 5252767 | 9265 | 5243502 | 5244815 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5245044 | 5245901 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5245904 | 5246284 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5246281 | 5246565 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5246658 | 5247089 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5247297 | 5248226 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5248376 | 5248915 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5248975 | 5249724 | putative antirepressor |
| 5243502 | 5252767 | 9265 | 5250264 | 5250878 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5251001 | 5251723 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5251873 | 5252238 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5252351 | 5252527 | hypothetical protein |
| 5243502 | 5252767 | 9265 | 5252555 | 5252767 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5248975 | 5249724 | putative antirepressor |
| 5248975 | 5269348 | 20373 | 5250264 | 5250878 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5251001 | 5251723 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5251873 | 5252238 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5252351 | 5252527 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5252555 | 5252767 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5253731 | 5254465 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5254462 | 5255052 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5255239 | 5255595 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5255749 | 5256153 | Phage peptidoglycan hydrolase |
| 5248975 | 5269348 | 20373 | 5256153 | 5256557 | hypothetical protein |

|  |  |  |  |  |  |
|---|---|---|---|---|---|
| 5248975 | 5269348 | 20373 | 5256561 | 5256755 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5256786 | 5257220 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5257198 | 5258691 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5258692 | 5258949 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5258949 | 5259689 | Phage protein |
| 5248975 | 5269348 | 20373 | 5259686 | 5261812 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5261802 | 5262038 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5262078 | 5263043 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5263070 | 5264059 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5264119 | 5264325 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5264322 | 5264642 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5264642 | 5265214 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5265211 | 5265819 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5265809 | 5267434 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5267431 | 5267883 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5267876 | 5269348 | hypothetical protein |
| 5248975 | 5269348 | 20373 | 5269345 | 5270595 | Phage capsid and scaffold |
| 5926066 | 5930102 | 4036 | 5926066 | 5927403 | hypothetical protein |
| 5926066 | 5930102 | 4036 | 5927534 | 5928142 | hypothetical protein |
| 5926066 | 5930102 | 4036 | 5928265 | 5928720 | Histone acetyltransferase HPA2 |
| 5926066 | 5930102 | 4036 | 5928973 | 5929086 | hypothetical protein |
| 5926066 | 5930102 | 4036 | 5929452 | 5930102 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5927534 | 5928142 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5928265 | 5928720 | Histone acetyltransferase HPA2 |
| 5927534 | 5940226 | 12692 | 5928973 | 5929086 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5929452 | 5930102 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5930245 | 5930862 | Transporter, LysE family |

| | | | | | |
|---|---|---|---|---|---|
| 5927534 | 5940226 | 12692 | 5931004 | 5931768 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5931905 | 5932438 | Isochorismatase (EC 3.3.2.1) |
| 5927534 | 5940226 | 12692 | 5932615 | 5933208 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5933337 | 5933882 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5934019 | 5934291 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5934873 | 5935730 | Mobile element protein |
| 5927534 | 5940226 | 12692 | 5935769 | 5935915 | Mobile element protein |
| 5927534 | 5940226 | 12692 | 5936034 | 5936543 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5936697 | 5937308 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5937411 | 5937578 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5937575 | 5938426 | Transposase |
| 5927534 | 5940226 | 12692 | 5938441 | 5938725 | Transposase |
| 5927534 | 5940226 | 12692 | 5938744 | 5938965 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5939242 | 5939598 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5939656 | 5939847 | hypothetical protein |
| 5927534 | 5940226 | 12692 | 5939930 | 5940226 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6014116 | 6015111 | Phage antirepressor protein |
| 6015101 | 6037387 | 22286 | 6015101 | 6015424 | FIG01206055: hypothetical protein |
| 6015101 | 6037387 | 22286 | 6015753 | 6016364 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6016364 | 6017059 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6017198 | 6017338 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6017410 | 6017856 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6017945 | 6018427 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6018578 | 6018910 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6019140 | 6019505 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6019480 | 6019794 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6015101 | 6037387 | 22286 | 6019823 | 6020035 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6020365 | 6020865 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6021027 | 6021599 | conserved hypothetical protein |
| 6015101 | 6037387 | 22286 | 6021599 | 6022237 | conserved hypothetical protein |
| 6015101 | 6037387 | 22286 | 6022512 | 6023102 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6023283 | 6023639 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6023793 | 6024197 | Phage peptidoglycan hydrolase |
| 6015101 | 6037387 | 22286 | 6024197 | 6024601 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6024605 | 6024799 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6024827 | 6025261 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6025239 | 6026732 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6026733 | 6026990 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6026990 | 6027730 | Phage protein |
| 6015101 | 6037387 | 22286 | 6027727 | 6029853 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6029843 | 6030079 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6030119 | 6031084 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6031111 | 6032100 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6032160 | 6032366 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6032363 | 6032683 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6032683 | 6033255 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6033252 | 6033860 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6034043 | 6035473 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6035470 | 6035922 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6035915 | 6037387 | hypothetical protein |
| 6015101 | 6037387 | 22286 | 6037384 | 6038595 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6014116 | 6015111 | Phage antirepressor protein |

| | | | | | |
|---|---|---|---|---|---|
| 6015101 | 6020865 | 5764 | 6015101 | 6015424 | FIG01206055: hypothetical protein |
| 6015101 | 6020865 | 5764 | 6015753 | 6016364 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6016364 | 6017059 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6017198 | 6017338 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6017410 | 6017856 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6017945 | 6018427 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6018578 | 6018910 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6019140 | 6019505 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6019480 | 6019794 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6019823 | 6020035 | hypothetical protein |
| 6015101 | 6020865 | 5764 | 6020365 | 6020865 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6145274 | 6145498 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6145491 | 6145868 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6145865 | 6146320 | FIG01200741: hypothetical protein |
| 6145491 | 6157422 | 11931 | 6146320 | 6146811 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6146798 | 6147454 | FIG01203863: hypothetical protein |
| 6145491 | 6157422 | 11931 | 6147530 | 6147700 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6147710 | 6148564 | Outer membrane receptor protein |
| 6145491 | 6157422 | 11931 | 6148574 | 6148840 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6148844 | 6148999 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6148996 | 6149184 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6149181 | 6149603 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6149587 | 6150072 | Methyl-accepting chemotaxis protein |
| 6145491 | 6157422 | 11931 | 6150073 | 6150786 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6145491 | 6157422 | 11931 | 6150789 | 6151940 | FIG01201088: hypothetical protein |
| 6145491 | 6157422 | 11931 | 6151988 | 6152395 | Structural protein P5 |
| 6145491 | 6157422 | 11931 | 6152392 | 6152490 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6152487 | 6152897 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6152897 | 6153064 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6153112 | 6153411 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6153825 | 6155318 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6155319 | 6155750 | hypothetical protein |
| 6145491 | 6157422 | 11931 | 6156058 | 6157422 | Transposase |
| 6146320 | 6150786 | 4466 | 6146320 | 6146811 | hypothetical protein |
| 6146320 | 6150786 | 4466 | 6146798 | 6147454 | FIG01203863: hypothetical protein |
| 6146320 | 6150786 | 4466 | 6147530 | 6147700 | hypothetical protein |
| 6146320 | 6150786 | 4466 | 6147710 | 6148564 | Outer membrane receptor protein |
| 6146320 | 6150786 | 4466 | 6148574 | 6148840 | hypothetical protein |
| 6146320 | 6150786 | 4466 | 6148844 | 6148999 | hypothetical protein |
| 6146320 | 6150786 | 4466 | 6148996 | 6149184 | hypothetical protein |
| 6146320 | 6150786 | 4466 | 6149181 | 6149603 | hypothetical protein |
| 6146320 | 6150786 | 4466 | 6149587 | 6150072 | Methyl-accepting chemotaxis protein |
| 6146320 | 6150786 | 4466 | 6150073 | 6150786 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6226150 | 6226485 | tRNA 2-thiouridine synthesis protein TusE |
| 6226150 | 6256311 | 30161 | 6226493 | 6226765 | Acylphosphate phosphohydrolase (EC 3.6.1.7) |
| 6226150 | 6256311 | 30161 | 6226853 | 6228256 | N-acetylglucosamine regulated methyl-accepting chemotaxis protein |

|  |  |  |  |  |  |
|---|---|---|---|---|---|
| 6226150 | 6256311 | 30161 | 6228376 | 6229572 | 23S rRNA (cytosine(1962)-C(5))-methyltransferase (EC 2.1.1.191) |
| 6226150 | 6256311 | 30161 | 6229625 | 6234196 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6235199 | 6235954 | Nucleoside permease NupC |
| 6226150 | 6256311 | 30161 | 6236489 | 6236869 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6236891 | 6237382 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6237843 | 6238730 | Transcriptional regulator, LysR family |
| 6226150 | 6256311 | 30161 | 6238854 | 6240404 | Alpha-1,2-mannosidase |
| 6226150 | 6256311 | 30161 | 6240597 | 6240710 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6241055 | 6241438 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6241571 | 6241789 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6242049 | 6242165 | Mobile element protein |
| 6226150 | 6256311 | 30161 | 6242377 | 6242616 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6242616 | 6242798 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6242805 | 6243188 | Transposase |
| 6226150 | 6256311 | 30161 | 6243185 | 6244168 | Transposase |
| 6226150 | 6256311 | 30161 | 6244618 | 6244800 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6244797 | 6245000 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6245117 | 6245791 | Transposase |
| 6226150 | 6256311 | 30161 | 6246099 | 6246431 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6246428 | 6246913 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6248147 | 6249301 | Transposase |
| 6226150 | 6256311 | 30161 | 6249462 | 6251738 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6252130 | 6252951 | Transposase InsO for insertion sequence element IS911 |
| 6226150 | 6256311 | 30161 | 6253013 | 6253291 | Transposase InsN for insertion sequence element IS911 |

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|---|---|---|---|---|---|
| 6226150 | 6256311 | 30161 | 6253690 | 6253851 | hypothetical protein |
| 6226150 | 6256311 | 30161 | 6253841 | 6255358 | deoxycytidylate deaminase-related protein |
| 6226150 | 6256311 | 30161 | 6256084 | 6256311 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6276544 | 6276708 | Mobile element protein |
| 6276544 | 6289853 | 13309 | 6276932 | 6278158 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6278151 | 6279029 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6279170 | 6279454 | Transposase |
| 6276544 | 6289853 | 13309 | 6279469 | 6280320 | Transposase |
| 6276544 | 6289853 | 13309 | 6280348 | 6282873 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6282866 | 6283513 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6283516 | 6284943 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6285111 | 6285944 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6286099 | 6286272 | Transposase |
| 6276544 | 6289853 | 13309 | 6287071 | 6289092 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6289281 | 6289853 | hypothetical protein |
| 6276544 | 6289853 | 13309 | 6289850 | 6291019 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6276932 | 6278158 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6278151 | 6279029 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6279170 | 6279454 | Transposase |
| 6276932 | 6298443 | 21511 | 6279469 | 6280320 | Transposase |
| 6276932 | 6298443 | 21511 | 6280348 | 6282873 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6282866 | 6283513 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6283516 | 6284943 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6285111 | 6285944 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6286099 | 6286272 | Transposase |
| 6276932 | 6298443 | 21511 | 6287071 | 6289092 | hypothetical protein |

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|---|---|---|---|---|---|
| 6276932 | 6298443 | 21511 | 6289281 | 6289853 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6289850 | 6291019 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6292328 | 6295174 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6295266 | 6297875 | hypothetical protein |
| 6276932 | 6298443 | 21511 | 6298297 | 6298443 | hypothetical protein |
| 6393492 | 6402737 | 9245 | 6393492 | 6394592 | hypothetical protein |
| 6393492 | 6402737 | 9245 | 6394579 | 6395670 | N-acetyl-D-glucosamine ABC transporter, permease protein 1 |
| 6393492 | 6402737 | 9245 | 6395673 | 6396542 | ABC transporter, permease protein 2 (cluster 1, maltose/g3p/polyamine/iron) |
| 6393492 | 6402737 | 9245 | 6396613 | 6397896 | N-acetyl-D-glucosamine ABC transporter, substrate-binding protein |
| 6393492 | 6402737 | 9245 | 6398166 | 6398429 | hypothetical protein |
| 6393492 | 6402737 | 9245 | 6398758 | 6399549 | hypothetical protein |
| 6393492 | 6402737 | 9245 | 6400865 | 6401962 | Alcohol dehydrogenase (EC 1.1.1.1) |
| 6393492 | 6402737 | 9245 | 6402567 | 6402737 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6428672 | 6429352 | NAD(P)H-flavin reductase (EC 1.5.1.29) (EC 1.16.1.3) |
| 6428672 | 6463385 | 34713 | 6429510 | 6429734 | Inorganic pyrophosphatase/exopolyphosphatase |
| 6428672 | 6463385 | 34713 | 6430649 | 6430855 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6431061 | 6432293 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6432436 | 6433695 | AmpG protein |
| 6428672 | 6463385 | 34713 | 6433692 | 6434720 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6434732 | 6436756 | TonB-dependent receptor |
| 6428672 | 6463385 | 34713 | 6437025 | 6437867 | Transcriptional regulator, AraC family |

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|---|---|---|---|---|---|
| 6428672 | 6463385 | 34713 | 6438258 | 6439004 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6438962 | 6439882 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6440121 | 6440408 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6440592 | 6441134 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6441155 | 6441691 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6441896 | 6442156 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6442156 | 6442431 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6442482 | 6442739 | putative DNA helicase |
| 6428672 | 6463385 | 34713 | 6442845 | 6443645 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6443768 | 6444064 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6444871 | 6446598 | FIG005666: putative helicase |
| 6428672 | 6463385 | 34713 | 6446601 | 6448550 | putative DNA helicase |
| 6428672 | 6463385 | 34713 | 6448928 | 6449191 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6449192 | 6449317 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6449389 | 6449997 | Transposase |
| 6428672 | 6463385 | 34713 | 6450324 | 6450503 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6450569 | 6450682 | Mobile element protein |
| 6428672 | 6463385 | 34713 | 6451854 | 6453428 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6453897 | 6454349 | Transposase InsO for insertion sequence element IS911 |
| 6428672 | 6463385 | 34713 | 6454368 | 6454550 | Transposase InsO for insertion sequence element IS911 |
| 6428672 | 6463385 | 34713 | 6454690 | 6454806 | Thiol peroxidase, Bcp-type (EC 1.11.1.15) |
| 6428672 | 6463385 | 34713 | 6455040 | 6455375 | HipB protein @ Antitoxin HigA |
| 6428672 | 6463385 | 34713 | 6455368 | 6456774 | Toxin HigB / Protein kinase domain of HipA |
| 6428672 | 6463385 | 34713 | 6456797 | 6457636 | Transposase InsO for insertion sequence element IS911 |

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|---|---|---|---|---|---|
| 6428672 | 6463385 | 34713 | 6457645 | 6457959 | Transposase InsN for insertion sequence element IS911 |
| 6428672 | 6463385 | 34713 | 6458264 | 6459592 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6459724 | 6460224 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6460344 | 6460727 | hypothetical protein |
| 6428672 | 6463385 | 34713 | 6461031 | 6463385 | hypothetical protein |
| 6438962 | 6446598 | 7636 | 6438258 | 6439004 | Mobile element protein |
| 6438962 | 6446598 | 7636 | 6438962 | 6439882 | Mobile element protein |
| 6438962 | 6446598 | 7636 | 6440121 | 6440408 | Mobile element protein |
| 6438962 | 6446598 | 7636 | 6440592 | 6441134 | Mobile element protein |
| 6438962 | 6446598 | 7636 | 6441155 | 6441691 | Mobile element protein |
| 6438962 | 6446598 | 7636 | 6441896 | 6442156 | hypothetical protein |
| 6438962 | 6446598 | 7636 | 6442156 | 6442431 | hypothetical protein |
| 6438962 | 6446598 | 7636 | 6442482 | 6442739 | putative DNA helicase |
| 6438962 | 6446598 | 7636 | 6442845 | 6443645 | hypothetical protein |
| 6438962 | 6446598 | 7636 | 6443768 | 6444064 | Mobile element protein |
| 6438962 | 6446598 | 7636 | 6444871 | 6446598 | FIG005666: putative helicase |
| 6486978 | 6508225 | 21247 | 6486978 | 6487538 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6487621 | 6490440 | IncF plasmid conjugative transfer protein TraG |
| 6486978 | 6508225 | 21247 | 6490437 | 6491858 | IncF plasmid conjugative transfer pilus assembly protein TraH |
| 6486978 | 6508225 | 21247 | 6491855 | 6492259 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6492272 | 6493030 | IncF plasmid conjugative transfer pilus assembly protein TraF |
| 6486978 | 6508225 | 21247 | 6493027 | 6494754 | IncF plasmid conjugative transfer protein TraN |
| 6486978 | 6508225 | 21247 | 6494751 | 6495329 | IncF plasmid conjugative transfer protein TrbC |

| | | | | | |
|---|---|---|---|---|---|
| 6486978 | 6508225 | 21247 | 6495340 | 6496344 | IncF plasmid conjugative transfer pilus assembly protein TraU |
| 6486978 | 6508225 | 21247 | 6496341 | 6497012 | IncF plasmid conjugative transfer pilus assembly protein TraW |
| 6486978 | 6508225 | 21247 | 6497009 | 6497218 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6497215 | 6499806 | IncF plasmid conjugative transfer pilus assembly protein TraC |
| 6486978 | 6508225 | 21247 | 6499809 | 6500210 | IncF plasmid conjugative transfer pilus assembly protein TraV |
| 6486978 | 6508225 | 21247 | 6500207 | 6501694 | IncF plasmid conjugative transfer pilus assembly protein TraB |
| 6486978 | 6508225 | 21247 | 6501684 | 6502424 | IncF plasmid conjugative transfer pilus assembly protein TraK |
| 6486978 | 6508225 | 21247 | 6502421 | 6502990 | IncF plasmid conjugative transfer pilus assembly protein TraE |
| 6486978 | 6508225 | 21247 | 6503003 | 6503305 | IncF plasmid conjugative transfer pilus assembly protein TraL |
| 6486978 | 6508225 | 21247 | 6503324 | 6503620 | IncF plasmid conjugative transfer pilin protein TraA |
| 6486978 | 6508225 | 21247 | 6504116 | 6504709 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6505056 | 6505187 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6505530 | 6506105 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6506105 | 6506746 | conserved hypothetical protein |
| 6486978 | 6508225 | 21247 | 6507224 | 6507457 | Mobile element protein |
| 6486978 | 6508225 | 21247 | 6507562 | 6507888 | Mobile element protein |
| 6486978 | 6508225 | 21247 | 6507980 | 6508093 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6508097 | 6508225 | hypothetical protein |
| 6486978 | 6508225 | 21247 | 6508222 | 6508635 | Antitoxin 1 |
| 6491855 | 6499806 | 7951 | 6490437 | 6491858 | IncF plasmid conjugative transfer pilus assembly protein TraH |
| 6491855 | 6499806 | 7951 | 6491855 | 6492259 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6491855 | 6499806 | 7951 | 6492272 | 6493030 | IncF plasmid conjugative transfer pilus assembly protein TraF |
| 6491855 | 6499806 | 7951 | 6493027 | 6494754 | IncF plasmid conjugative transfer protein TraN |
| 6491855 | 6499806 | 7951 | 6494751 | 6495329 | IncF plasmid conjugative transfer protein TrbC |
| 6491855 | 6499806 | 7951 | 6495340 | 6496344 | IncF plasmid conjugative transfer pilus assembly protein TraU |
| 6491855 | 6499806 | 7951 | 6496341 | 6497012 | IncF plasmid conjugative transfer pilus assembly protein TraW |
| 6491855 | 6499806 | 7951 | 6497009 | 6497218 | hypothetical protein |
| 6491855 | 6499806 | 7951 | 6497215 | 6499806 | IncF plasmid conjugative transfer pilus assembly protein TraC |
| 6534932 | 6550490 | 15558 | 6534932 | 6535111 | Transposase and inactivated derivatives |
| 6534932 | 6550490 | 15558 | 6535191 | 6535799 | Transposase and inactivated derivatives |
| 6534932 | 6550490 | 15558 | 6536737 | 6536958 | Mobile element protein |
| 6534932 | 6550490 | 15558 | 6537434 | 6538948 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6538948 | 6539187 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6539169 | 6540080 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6540092 | 6540868 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6540868 | 6542091 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6542104 | 6542925 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6542915 | 6543748 | Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23) |
| 6534932 | 6550490 | 15558 | 6543738 | 6544343 | Thymidylate kinase (EC 2.7.4.9) |
| 6534932 | 6550490 | 15558 | 6544336 | 6544728 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6544754 | 6545050 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6545300 | 6546457 | Cysteine desulfurase (EC 2.8.1.7) |
| 6534932 | 6550490 | 15558 | 6546467 | 6547165 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6534932 | 6550490 | 15558 | 6547210 | 6547551 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6547548 | 6548876 | hypothetical protein |
| 6534932 | 6550490 | 15558 | 6549585 | 6550490 | 4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7) |
| 6544754 | 6550490 | 5736 | 6544754 | 6545050 | hypothetical protein |
| 6544754 | 6550490 | 5736 | 6545300 | 6546457 | Cysteine desulfurase (EC 2.8.1.7) |
| 6544754 | 6550490 | 5736 | 6546467 | 6547165 | hypothetical protein |
| 6544754 | 6550490 | 5736 | 6547210 | 6547551 | hypothetical protein |
| 6544754 | 6550490 | 5736 | 6547548 | 6548876 | hypothetical protein |
| 6544754 | 6550490 | 5736 | 6549585 | 6550490 | 4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7) |
| 6562799 | 6578149 | 15350 | 6561489 | 6562805 | Toxin HigB / Protein kinase domain of HipA |
| 6562799 | 6578149 | 15350 | 6562799 | 6563149 | transcriptional regulator, Cro/CI family |
| 6562799 | 6578149 | 15350 | 6563892 | 6564059 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6564112 | 6564570 | Retron-type RNA-directed DNA polymerase (EC 2.7.7.49) |
| 6562799 | 6578149 | 15350 | 6564829 | 6564984 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6564971 | 6566200 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6566334 | 6566543 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6566557 | 6566958 | VapC toxin protein |
| 6562799 | 6578149 | 15350 | 6566972 | 6567205 | VapB protein (antitoxin to VapC) |
| 6562799 | 6578149 | 15350 | 6567355 | 6567471 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6567516 | 6568769 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6568899 | 6569579 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6569843 | 6570451 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6570814 | 6571212 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6571209 | 6571484 | hypothetical protein |

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|---|---|---|---|---|---|
| 6562799 | 6578149 | 15350 | 6571521 | 6571721 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6572282 | 6572596 | ParE toxin protein |
| 6562799 | 6578149 | 15350 | 6572589 | 6572831 | ParD protein (antitoxin to ParE) |
| 6562799 | 6578149 | 15350 | 6573460 | 6573696 | Site-specific recombinase, phage integrase family |
| 6562799 | 6578149 | 15350 | 6573782 | 6574534 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6574545 | 6575594 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6575584 | 6576285 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6576295 | 6576576 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6576586 | 6577197 | hypothetical protein |
| 6562799 | 6578149 | 15350 | 6577283 | 6578149 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6605904 | 6606560 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6606557 | 6607627 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6607605 | 6608006 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6608090 | 6609130 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6609202 | 6609657 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6609824 | 6610981 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6610996 | 6612186 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6612280 | 6613353 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6613357 | 6613911 | hypothetical protein |
| 6605904 | 6613911 | 8007 | 6613908 | 6614642 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6620431 | 6621372 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6621398 | 6622087 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6622080 | 6623696 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6623758 | 6623880 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6624017 | 6624544 | Transcriptional regulator, PadR family |
| 6620431 | 6629666 | 9235 | 6624697 | 6625731 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6620431 | 6629666 | 9235 | 6626006 | 6626122 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6626264 | 6626467 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6626481 | 6626879 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6627006 | 6627311 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6627321 | 6627518 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6627577 | 6627861 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6627952 | 6628212 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6628224 | 6628439 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6628456 | 6628665 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6628667 | 6628963 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6628974 | 6629351 | hypothetical protein |
| 6620431 | 6629666 | 9235 | 6629370 | 6629666 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6653695 | 6654441 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6654533 | 6654730 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6654816 | 6655292 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6655343 | 6655702 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6655834 | 6656100 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6656234 | 6656488 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6656829 | 6657284 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6657306 | 6660680 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6661049 | 6662053 | hypothetical protein |

| 6653695 | 6754393 | 100698 | 6662065 | 6662382 | hypothetical protein |
|---|---|---|---|---|---|
| 6653695 | 6754393 | 100698 | 6662586 | 6662993 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6663134 | 6663844 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6664038 | 6665279 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6665334 | 6665765 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6665758 | 6666435 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6666539 | 6666775 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6666826 | 6667023 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6667078 | 6667209 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6667257 | 6667949 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6668144 | 6668671 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6668989 | 6669381 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6669374 | 6669640 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6669655 | 6669933 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6669973 | 6670098 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6670309 | 6671307 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6672523 | 6674184 | DNA primase DnaG |

| | | | | | |
|---|---|---|---|---|---|
| 6653695 | 6754393 | 100698 | 6674186 | 6675115 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6675265 | 6676731 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6676743 | 6679181 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6679250 | 6679768 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6679765 | 6680154 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6680166 | 6682709 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6682784 | 6683746 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6683743 | 6684762 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6684773 | 6685687 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6685684 | 6686715 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6686735 | 6688609 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6688629 | 6689153 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6689202 | 6689795 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6689812 | 6689967 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6689970 | 6690512 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6690514 | 6691956 | TrbA-like protein |
| 6653695 | 6754393 | 100698 | 6691943 | 6692569 | hypothetical protein |

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|---|---|---|---|---|---|
| 6653695 | 6754393 | 100698 | 6692571 | 6693143 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6693154 | 6693516 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6693516 | 6694103 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6694181 | 6694855 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6695039 | 6697255 | DNA topoisomerase I (EC 5.99.1.2) |
| 6653695 | 6754393 | 100698 | 6697266 | 6697574 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6697579 | 6703122 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6703182 | 6704738 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6704832 | 6706343 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6706323 | 6706904 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6706974 | 6707603 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6707603 | 6707893 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6707901 | 6708212 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6708213 | 6710642 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6710743 | 6711492 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6711575 | 6712681 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6712817 | 6713140 | hypothetical protein |

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|---|---|---|---|---|---|
| 6653695 | 6754393 | 100698 | 6713122 | 6713829 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6713902 | 6716337 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6716703 | 6718430 | ATP-dependent DNA helicase RecQ |
| 6653695 | 6754393 | 100698 | 6718521 | 6719519 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6719751 | 6720482 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6720495 | 6720692 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6720722 | 6721057 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6721304 | 6722749 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6722771 | 6723643 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6723678 | 6724835 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6725044 | 6725190 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6725471 | 6726769 | Transposase |
| 6653695 | 6754393 | 100698 | 6726841 | 6727023 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6727023 | 6727262 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6727474 | 6728376 | Transposase |
| 6653695 | 6754393 | 100698 | 6728458 | 6729039 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6729065 | 6729271 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6653695 | 6754393 | 10069 8 | 6729292 | 6729495 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6729522 | 6729797 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6729794 | 6729964 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6730056 | 6730826 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6730845 | 6731012 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6731022 | 6731201 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6731214 | 6731423 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6731436 | 6731858 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6732248 | 6732445 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6732460 | 6733026 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6733055 | 6733399 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6733405 | 6733740 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6733777 | 6734136 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6734174 | 6734554 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6734570 | 6735112 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6735152 | 6735427 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6735408 | 6736412 | Putative DNA-binding protein in cluster with Type I restriction-modification system |

| | | | | | |
|---|---|---|---|---|---|
| 6653695 | 6754393 | 10069 8 | 6736421 | 6737074 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6737312 | 6738337 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6738787 | 6739566 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6739761 | 6740723 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6740711 | 6741070 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6741087 | 6741812 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6741799 | 6742221 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6742218 | 6743159 | ATP synthase gamma chain (EC 3.6.3.14) |
| 6653695 | 6754393 | 10069 8 | 6743463 | 6744212 | Error-prone repair protein UmuD |
| 6653695 | 6754393 | 10069 8 | 6744205 | 6745461 | Error-prone, lesion bypass DNA polymerase V (UmuC) |
| 6653695 | 6754393 | 10069 8 | 6745499 | 6746215 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6746220 | 6746621 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6746632 | 6747162 | diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s) |
| 6653695 | 6754393 | 10069 8 | 6747184 | 6747597 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6747668 | 6748831 | hypothetical protein |
| 6653695 | 6754393 | 10069 8 | 6748828 | 6750447 | Twitching motility protein PilT |

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|---|---|---|---|---|---|
| 6653695 | 6754393 | 100698 | 6750465 | 6751289 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6751298 | 6752752 | hypothetical protein |
| 6653695 | 6754393 | 100698 | 6752771 | 6754393 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6684773 | 6685687 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6685684 | 6686715 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6686735 | 6688609 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6688629 | 6689153 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6689202 | 6689795 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6689812 | 6689967 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6689970 | 6690512 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6690514 | 6691956 | TrbA-like protein |
| 6685684 | 6697574 | 11890 | 6691943 | 6692569 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6692571 | 6693143 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6693154 | 6693516 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6693516 | 6694103 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6694181 | 6694855 | hypothetical protein |
| 6685684 | 6697574 | 11890 | 6695039 | 6697255 | DNA topoisomerase I (EC 5.99.1.2) |
| 6685684 | 6697574 | 11890 | 6697266 | 6697574 | hypothetical protein |
| 6718521 | 6722749 | 4228 | 6718521 | 6719519 | hypothetical protein |
| 6718521 | 6722749 | 4228 | 6719751 | 6720482 | hypothetical protein |
| 6718521 | 6722749 | 4228 | 6720495 | 6720692 | hypothetical protein |
| 6718521 | 6722749 | 4228 | 6720722 | 6721057 | hypothetical protein |
| 6718521 | 6722749 | 4228 | 6721304 | 6722749 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6731214 | 6731423 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| 6731214 | 6737074 | 5860 | 6731436 | 6731858 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6732248 | 6732445 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6732460 | 6733026 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6733055 | 6733399 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6733405 | 6733740 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6733777 | 6734136 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6734174 | 6734554 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6734570 | 6735112 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6735152 | 6735427 | hypothetical protein |
| 6731214 | 6737074 | 5860 | 6735408 | 6736412 | Putative DNA-binding protein in cluster with Type I restriction-modification system |
| 6731214 | 6737074 | 5860 | 6736421 | 6737074 | hypothetical protein |
| 6746220 | 6750447 | 4227 | 6746220 | 6746621 | hypothetical protein |
| 6746220 | 6750447 | 4227 | 6746632 | 6747162 | diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s) |
| 6746220 | 6750447 | 4227 | 6747184 | 6747597 | hypothetical protein |
| 6746220 | 6750447 | 4227 | 6747668 | 6748831 | hypothetical protein |
| 6746220 | 6750447 | 4227 | 6748828 | 6750447 | Twitching motility protein PilT |

### 3.4.3.3 Presence of prophages

Prophages are the genetic material of a bacteriophage, incorporated into the genome of a bacterium and able to produce phages if specifically activated. To identify and annotate putative prophage sequences in the isolated strains, PHASTER web server was used. The results have showed that TUMSAT-V. nig1 consists with four phages PHAGE_Entero_mEP235 (15 CDSs), PHAGE_Sulfit_pCB2047- 2 copies (21 CDSs), and PHAGE_Entero_HK446 (21CDSs).

PHAGE_Vibrio_PV94 which is having 46 CDSs is only found in TUMSAT-V. nig2 and TUMSAT-V. nig3 strains (Table 3.5). Among the CDS, it has been found with tail sheath protein, long tail fiber distal subunit, phage repressor, methyltransferase type II along with numerous hypothetical proteins which may be responsible for the virulence of the isolated strains.

Table 3.5: Predicted prophages in the genomes of the three strains of *V. nigripulchritudo* using PHASTER

| Predicted phage | Location | Region length | # of total proteins | % GC |
|---|---|---|---|---|
| PHAGE_Entero_mEP235 | TUMSAT-V. nig1 | 30.7 | 13 | 43.70 |
| PHAGE_Sulfit_pCB2047 | TUMSAT-V. nig1 | 17.4 | 21 | 51.39 |
| PHAGE_Entero_HK446 | TUMSAT-V. nig1 | 26.2 | 19 | 44.50 |
| PHAGE_Vibrio_PV94 | TUMSAT-V. nig2 | 36.8 | 44 | 44.92 |
| | TUMSAT-V. nig3 | 37.3 | 44 | 44.96 |

### 3.4.4 Comparative genomic analysis of the isolated *V. nigripulchritudo* strains

#### 3.4.4.1 Distribution of orthologous genes

Orthologous genes usually retain functions similar with those of their ancestral genes. They can retain the same function during evolution. OrthoVenn2 web tool was used to identify the distribution of shared and unique orthologous gene clusters of the 03 strains of isolated *V. nigripulchritudo* (TUMSAT-V. nig1, TUMSAT-V. nig2, and TUMSAT-V. nig3) along with two reference genomes of *V. nigripulchritudo* available in gene bank. The used reference strains were *V. nigripulchritudo* TUMSAT-TG-2018 isolated from Japan in 2018 and *V. nigripulchritudo* SFn1-2013 isolated from New Caledonia in 2013.

After predicting the orthologous genes in these five genomes, 4,924 common orthologous clusters were found in all 5 strains responsible for 24,657 common proteins indicating a same evolutionary background. These common clusters are consisted with toxin biosynthesis, glutamate biosynthesis, proteolysi, transketolase activity, dTTP biosynthetic process, regulation of metanephric nephrone tubule epithelial cell differentiation, dicarboxylate transport and bacterial type flagellum dependent cell motility etc.

266 common clusters were found in the isolated strain of *V. nigripulchritudo* TUMSAT-V. nig1, TUMSAT-V. nig2, and TUMSAT-V. nig3 consisted with aromatic compound catabolic process, DNA replication, type IV pilus dependent motility, DNA integration, plasmid portioning, transposition, transferase activity, regulation of blood coagulation, nuclease activity, conjugation etc. The results have indicated that strains TUMSAT-V. nig2 and TUMSAT-V. nig3 are having the highest number of shared gene clusters (n=444) while TUMSAT-V. nig1 and TUMSAT-V. nig3 show the lowest shared gene cluster as n=6 (Figure 3.7).

Also, the unique gene cluster (n=50) found only in TUMSAT-V. nig1 presents with transposition, zinc ion transmembrane transporter activity, valine biosynthesis and ectonie biosynthesis, oxidation reduction process, glycine betaine biosynthetic process from choline, transposition, lipid metabolic process, NADP catabolic process, oxidoreductase activity, lignin catabolic process, benzoate catabolic process via hydroxylation, urate catabolic process, cellular response to nitric oxide etc.

The Venn diagram obtained from the OrthoVenn2 web tool indicates the distribution of shared and unique orthologous gene clusters among five *V. nigripulchritudo* strains. This also showed the presence of 4924 common orthologous clusters were found in all 05 strains followed by the highest homology in TUMSAT-V. nig2 and TUMSAT-V. nig3 being as the most closely related species (Figure 3.8).

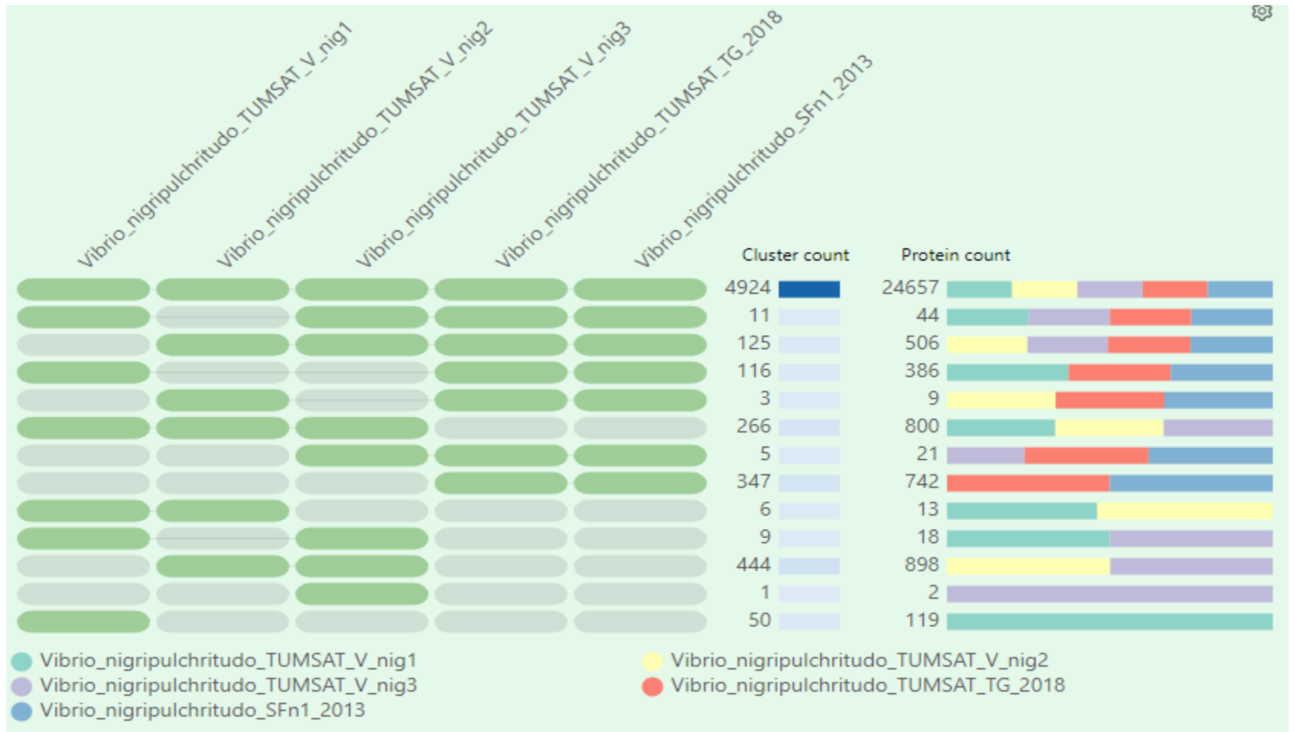


Figure 3.7: Shared proteins and clusters by the strains of *V. nigripulchritudo* TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3, TUMSAT-TG-2018, SFn1-2013

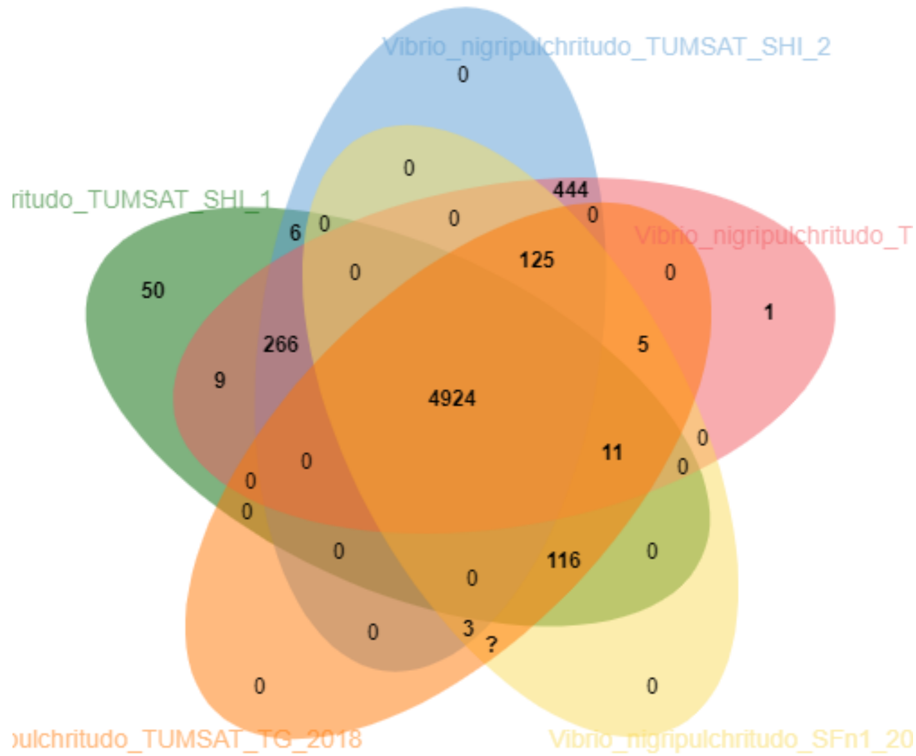


Figure 3.8: Venn diagram showing the distribution of shared and unique orthologous gene clusters among five *V. nigripulchritudo* strains

#### 3.4.4.3 Phylogenetic tree reconstruction

For the whole genome comparison, a phylogenetic analysis was conducted based on whole-genome proteome data of *V. nigripulchritudo* genomes using Type Strain Genome Server with iTOL. For the reconstruction of the phylogenetic tree, 03 strains of isolated *V. nigripulchritudo* (TUMSAT-V. nig1, TUMSAT-V. nig2, and TUMSAT-V. nig3) along with two reference genomes of *V. nigripulchritudo* (TUMSAT-TG-2018 and SFn1-2013) were used.

The resulting tree split the *V. nigripulchritudo* strains into three clusters. Cluster I consisted of strains TUMSAT-V. nig1 and TUMSAT-TG-2018 (reference strain isolated from Japan) while the cluster 2 is with SFn1-2013 strains of the V. nigripulchritudo (reference strain isolated from New Caledonia). TUMSAT-V. nig2 and TUMSAT-V. nig3 are in the cluster 3 showing the higher homology in between these strains. These results are correlated with the findings of Orthovenn2 as there also much more similarity was observed with common orthologs gene clusters for TUMSAT-V. nig2 and TUMSAT-V. nig3 (Figure 3.9).

Also, the phylogenetic tree has indicated that the *V. nigripulchritudo* are belonged to the same clade as *V. penaeicida* which corresponds to the findings of Kawato *et al*., 2020.

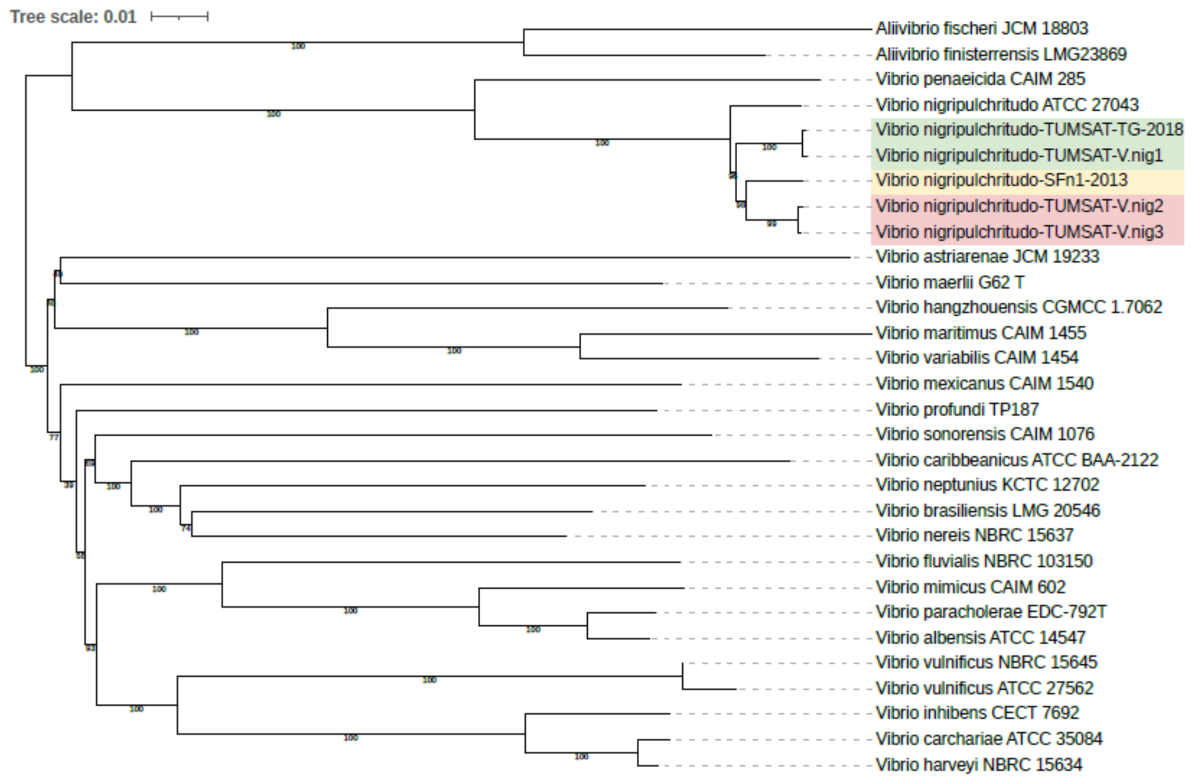


Figure 3.9: Phylogenetic tree of *V. nigripulchritudo* strains based on whole-genome proteome data

### 3.5 Discussion

Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. A new revolution has begun on bacterial diseases with the onset of bacterial whole genome sequencing for the discovery of the virulence related genes involved in pathogenicity of respective microbial pathogens. Therefore, the study of genes and their functions, host-pathogen interactions and comparative genomics with other related species would allow a complete understanding on pathogenicity and virulence of *V. nigripulchritudo.*

The genome sequencing was performed using Illumine MiSeq and Naopore GridION platform to get a better insight about their genomic feature. Illumina MiSeq sequencing platform is a group of cyclic-array sequencing technology belonged to the second-generation platforms composed with the preparation and amplification of libraries (prepared from DNA/RNA samples), clonal expansion, sequencing, and analysis (Shendure and Ji, 2008). Using short read data of Illumina sequencing alone will produce a complete genome for *de novo* assembly, but in pieces as a draft genome with the inability to deal with the repetitive regions (Boolchandani *et al*., 2019). For the genome to be assembled into a single chromosome with the presence of plasmids, reads would need to be longer than the longest repeated element on the genome usually ~7,000 base pairs.

To eliminate the limitations of PCR-based methods, such as nucleotide misincorporation by a polymerase, chimera formation and allelic dropouts (preferential amplification of one allele) causing an artificial homozygosity call, the introduction of the third generation NGS technology was brought. Sequencing data obtained from Nanopore assembly (commonly >40,000 bp) can fully span the repeats while revealing the arrangement of the all the genomic fragments of the genome enabling the correct structural resolution of complex genome regions (Ruan *et al*., 2020). However, the bacterial genome assemblies made using long reads alone are not adequate to get a better insight about their genomic features due to the high rate of errors in the raw sequence reads and the generation of artefacts (Senol Cali *et al*., 2019).

Therefore, a standard new approach has begun regarding the combination of the use of short and long-read sequencing technologies for obtaining high-quality assemblies for studying the genomic characterization of bacterial genomes. For our study based on the identification and characterization of the genomic features of the isolated strains of *V. nigripulchritudo*, the hybrid assemblies were made using the sequence data obtained from Illumina and nanopore as the long

reads provide information on the genome structure while the short reads provide high base-level accuracy.

The obtained genomic data from the hybrid assemblies have indicated that all the isolated strains of *V. nigripulchritudo* are found with two chromosomes (chromosome I and chromosome II) which is which is consistent with the results from other *Vibrio* species. All known *Vibrio* sp. are having two chromosomes in their genomes (Lukjancenko and Usseryibrio, 2014). The presence of two chromosomes in *Vibrio* genomes was first revealed by Trucksis *et al*. (1998) indicating the presence of the two chromosomes in *V. cholerae*. Usually, the chromosome I is the larger one with a relatively constant size of 4 million base pairs while the chromosome II is comparatively smaller with around 1-2 million base pairs (Rowe-Magnus *et al*., 1999). Our findings of the genome assemblies of the isolated strains are corelated with that as the sizes of both chromosomes were relatively constant among the isolated strains of *V. nigripulchritudo* genomes. Chromosome I size ranged from 4.02 to 4.07 Mb and chromosome II sizes ranged from 2.16 to 2.18 Mb for all the three strains of *V. nigripulchritudo.*

There are multiple discussions have been made about the purpose and the origin of the smaller chromosome in *Vibrio* genome as a characteristic feature. It has been assumed previously that; the chromosome II was originated as a megaplasmid. However, several researchers have suggested that the chromosome II play major roles in the genome to maintain the pathogenesis while optimizing the fast replication rate (Kirkup *et al*., 2010; Dikow and Smith, 2013).

In microbial ecology and evolution, plasmids have a great role as the carrier of horizontal gene transfer mechanism and as reservoirs of accessary gene functions in microbes (Tazzyman and Bonhoeffer 2013). In our study it has been found with two plasmids in *V. nigripulchritudo* TUMSAT-V. nig1 and only one plasmid with *V. nigripulchritudo* TUMSAT-V. nig 2 and TUMSAT-V. nig 3 respectively. However, the RAST analysis and the VFDB analysis indicate that there are no virulence related genes found in the plasmids of either of these genes. Instead of that the plasmids were occupied with the genes involved in their metabolism like membrane transportation, regulation and cell signaling, and metabolism.

The functional differences are observed among the genes which were horizontally transferred in the plasmid and those found at the core genome (Hacker and Carniel, 2001). As the plasmids replicate independently and can be transferred from either horizontally or vertically, this divergence of the functions is visualized with different evolutionary trajectories of plasmids and chromosomes (Werren, 2011).

The identified virulence related genes of the isolated *V. nigripulchritudo* strains were classified into seven categories including adherence, antiphagocytosis, chemotaxis and motility, iron uptake, quorum sensing, secretion system and toxins. All major virulence related genes are in either chromosome I or chromosome II in the genomes.

Adherence is an essential step in bacterial pathogenesis or infection, required for colonizing a new host. Adhesion occurs when adhesive molecules expressed on the bacterial surface bind to host surface receptors. Among the six genes related with the adhesion, four genes were associated for the Type IV pilus and two genes for accessory colonization factor and mannose-sensitive hemagglutinin (MSHA type IV pilus) in the isolated strains. The gene *acfB* is regulated by the same mechanism that controls the expression of cholera toxin and it exhibits sequence similarity with chemotaxis regulatory proteins found in another *Vibrio* sp. (Everiss *et al*., 1994). The presence of Type IV pilus is involved with the bio film formation of the *Vibrio* species (Paranjpye *et al*., 2005) and the presence of this gene in the genomes of *V. nigripulchritudo* governs the pathogenicity of them. 51 genes corelating *fil, che, fla, flg, flh, fli, flr,* and *mot* were identified for chemotaxis and motility for the flagellar structure and function in both chromosomes of *V. nigripulchritudo* while higher number of genes were found in chromosome I. It has been found that Flp pili play roles in host invasion, survival in the blood stream and resistance to complement activation in *V. vulnificus* (Duong-Nu *et al*., 2019). This same function would be occurred in the isolated *V. nigripulchritudo* too. In the chromosome I of the isolated strains, mannose- sensitive hemagglutinin is present. Surface motility and initial attachment is governed by the presence of mannose- sensitive hemagglutinin (MSHA type IV pilus) while playing a major role in biofilm formation which is considered as a crucial stage for surface selection, irreversible attachment, and ultimately microcolony formation (Teschler *et al*., 2015).

Presence of capsular polysaccharides related with the Antiphagocytosis is a major virulence factor present in pathogenic bacteria such as *V. cholerae, V. anguillarum* and other *Vibrio* related species. Here also, we identified five genes related with the presence of capsular polysaccharides which involved in Antiphagocytosis which is one of the key innate immune mechanisms of Penaeid shrimps.

The plasmid mediated iron uptake system is an important role as a virulence factor in most pathogenic Vibrio species (Stork *et al*., 2002). The chromosome mediated iron uptake system is identified in the isolated bacterial strains with the presence of Vibriobactin, siderophore that

helps the microbial system to acquire iron, Enterobactin receptors and Heme receptors governing the pathogenesis related to iron uptake.

In most of the *Vibrio* species, hemolysin is considered as one of the major pathogenic factors showing hemolytic activity against erythrocytes by manifesting either wound infection or intestinal infection as their clinical symptom. Several studies have reported that Vibrio species such as *V. mimicus, V.vulnificus*, and *V. fluvialis* produce hemolysin that shares some common structural features with HlyA (Kothary *et al*., 2003). In the isolated *V. nigripulchritudo*, it also found with thermolabile hemolysin (tlh) in the chromosome II of their genome indicating the ability to cause the hemolytic activity in the infected shrimps. The findings are corelated with the studies in *V. cholerae* (Xu et al., 2019) and *V. harveyi* (Deng *et al*., 2019).

Quorum-sensing allows individual bacteria within colonies to coordinate and carry out colony-wide functions such as: sporulation, bioluminescence, virulence, conjugation, competence, and biofilm formation (Turovskiy *et al*., 2007). In the present study, two types of virulence related genes for quorum sensing were identified as luxS for auto inducer -2 and cqsA for cholera autoinducer 1 respectively Those were distributed in both chromosomes of the *V. nigripulchritudo* genome. As stated by Prithvisagar *et al*. (2021), cqsA and luxS genes have also been recently reported in *V. parahaemolyticus* associated with disease outbreak in shrimp.

In the pathogenic bacteria, the extracellular protein secretion (EPS) type II secretion system is mediated by the genes epsC–epsN. It has been reported that, these genes are essential for secretion of the cholera toxin (Abendroth *et al*., 2009). The genes related for type II secretion system are found in the chromosome I of *V. penaeicida* (Ragab *et al*., 2022). In our study also, the same genes were in chromosome I. Simultaneously, other secretion systems including VAS type VI secretion system with VAS effector proteins are in chromosome II of the isolated strains which were reported as capable of injecting lethal toxins (Hood *et al*., 2010).

Since the 1960’s, issues associated with antibiotic resistant bacteria (ARB) have considered as a critical problem in the aquaculture industry (Nathan and Cars, 2014). Antimicrobial resistance (AMR) happens when some of the bacterial cells express with anti-microbial resistance genes in their genome. So that, they can develop the ability to defeat the drugs designed to kill them while continuing to grow causing severe environmental threat with the spreading of AMR genes (Rossolini *et al*., 2014). In previous literature, it has been found that to eradicate V vulnificus infection, tetracycline, third generation cephalosporins, and imipenem are used as effective antibiotics (Yun and Kim, 2018). In the present study, several antimicrobial resistance genes were identified which were creating resistance towards Macrolide, Fluroquinolone, Penam,

Tetracycline, Cephalosporin and Cephamycin with strict hits. Although the antibiotic usage is reduced for shrimp aquaculture in most of the major farming countries, still it would create a harmful effect as it has been revealed that tetracycline resistance genes (tet) are retained in sediments of aquaculture sites even after fish culture has stopped for several years, suggesting that the sediments are an ARGs-reservoir in the natural environment (Tamminen *et al*., 2011).

Genomic islands (GIs) are the accessory genes acquired by horizontal transfer and recognized as discrete DNA segments between closely related strains. As the GIs are having a significant impact on the genome plasticity and evolution, they contribute to the diversification and adaptation of microorganisms with the dissemination of antibiotic resistance, virulence genes, and formation of catabolic pathways (Juhas *et al*., 2009). Horizontal gene transfer facilitated by GIs is having a significant role in role in the evolution of bacterial species which is attributed by simple acquisition and loss of GEI-borne genes and the possibility of GEIs transferring parts of a host's chromosomal DNA (Hochhut *et al*., 2000).

Presence of genomic islands indicate the evidence of the horizontal gene transfer events occurred in the isolated strains *V. nigripulchritudo*- TUMSAT-V. nig1 consists of the highest number of regions encoding GIs, suggesting that this strain has experienced numerous HGT events mediated by GIs. Also, some of the virulence factors are found in the GIs of V. nigripulchritudo- TUMSAT-V. nig1 including T1SS secreted agglutinin RTX, Flagellar protein, Hemolysin-related protein, T6SS PAAR-repeat protein and Toxin HigB / Protein kinase domain of HipA creating pathogenic islands. As stated by Hamilton *et al*. (2005), the presence of a wide variety of secretion systems in GIs would suggest that they can be used for the transferring of GIs and the host chromosomal DNA.

Toxin HigB / Protein kinase domain of HipA is found one of the genomic islands of *V. nigripulchritudo*. One prominent toxin/antitoxin (T/A) system found in many pathogens is HigB/HigA in which the antitoxin HigA masks the toxicity of the toxin HigB. Genes for the HigB/HigA in TA system are found on the plasmid originally isolated from *Proteus vulgaris* and later on it has been found in the chromosomes of other pathogens like *Vibrio cholera*, *Streptococcus pneumonia*, *Acinetobactor baumanni* etc. (Wood and Wood, 2016). Therefore, the presence of HigB/Hig A system suggests the horizontal gene transfer activities occurred in isolated *V. nigripulchritudo*. The presence of the Beta-lactamase class C-like and penicillin binding were identified in the GIs of respective *V. nigripulchritudo* would suggest that the antibiotic resistance gene would come from another *Vibrio* related species during the evolution of the genomes.

When the phages penetrate the bacterial plasma membrane and release genetic material into the cytoplasm, the viral components are encapsulated to form complete phages in the lytic life cycle (Canchaya *et al*. 2003). In contrast, the immediate replication and the releasing of the phages are not resulted in lysogenic lifecycle. Instead of that, the injected viral DNA named as prophage is integrated into the genome of the host and replicates as part of the bacterial chromosome while being as dormant until the lytic cycle is induced (Miller, 1981). Therefore, the presence of the prophages in a bacterial genome is good evidence that the bacteria has undergone the horizontal gene transfer activities in its evolutionary history. As it is believed that the prophages can increase the virulence potential of bacterial strains and able to increase the ability of the bacteria to survive in harsh environments, the higher number of prophages may enhance the virulence of TUMSAT-V. nig1 strain compared with the other isolated strains.

The comparative genomic analysis based on whole-genome proteome data of five *V. nigripulchritudo* genomes (three isolated strains with two reference genomes of *V. nigripulchritudo*) showed that the resulting tree split the *V. nigripulchritudo* strains into three clusters. The higher evolutionary homology is observed in the TUMSAT-V. nig1 and TUMSAT-TG-2018 and TUMSAT-V. nig2 and TUMSAT-V. nig3 respectively while the SFn1-2013 isolated from New Caledonia split into a separate cluster. These findings are corelated with the findings obtained from OrthVenn2 to show the distribution of shared and unique orthologous gene clusters among these five *V. nigripulchritudo* strains. Also, the phylogenetic tree has indicated that the *V. nigripulchritudo* are belonged to the same clade as *V. penaeicida* which corresponds to the findings of the previous literature (Ragab *et al*., 2022).

### 3.6 Conclusion

Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. Therefore, the Illumina and Nanopore whole genome sequencing technology was used to create hybrid genomic assemblies as the long reads provide information on the genome structure while the short reads provide high base-level accuracy. The genome assembly of the isolated strains of *V. nigripulchritudo* has revealed that all the strains are occupied with two chromosomes (chromosome I and chromosome II) with the presence of either one or two plasmids in their genomes.

Numerous virulence related genes are present in either chromosome I or chromosome II. Adherence is found with Mannose-sensitive hemagglutinin and Type IV pilus while antiphagocytosis is observed with capsular polysaccharides. Flagella are used to maintain chemotaxis and motility in the isolated strains. Iron uptake is governed with Vibriobactin, Enterobactin receptors and Heme receptors. For the quorum sensing, auto inducer-1 and cholera auto inducer-2 are found in chromosome I and chromosome II respectively. As bacterial secretion systems for secretion of substances as their virulence factors (mainly of proteins) to invade the host cells, the isolated strains are found with EPS type II secretion system and VAS type VI secretion system. Hemolysin gene is found in the chromosome II of the isolated strain as a potential virulence factor which causes lysis of red blood cells of the host.

Presence of genomic islands, prophages and antibiotic resistance genes have indicated the evidence of the horizontal gene transfer events occurred in the isolated strains *V. nigripulchritudo* with the acquisition new genetic information through HGT, which has a significant role in bacterial evolution and affects pathogenesis. The comparative genomic analysis based on whole-genome proteome data of five *V. nigripulchritudo* genomes (three isolated strains with two reference genomes of *V. nigripulchritudo*) showed that the resulting tree split the *V. nigripulchritudo* strains into three clusters. The higher evolutionary homology is observed in the TUMSAT-V. nig1 and TUMSAT-TG-2018 followed by TUMSAT-V. nig2 and TUMSAT-V. nig3 respectively while the SFn1-2013 isolated from New Caledonia split into a separate cluster

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# Chapter 04

## Summary and General Conclusion

The contribution of aquaculture is having a significant increase with 48% in 2019 when compared to the 26 % in 2000. Also, the aquaculture production reached 87.5 million tons which is 6% higher than in 2018. This scenario helps to overcome the global food insecurity and malnutrition while achieving the sustainable utilization of the marine and freshwater resources. With the steady growth of the global aquaculture production, contribution of the farmed crustaceans was 7.5% from the global production with a volume of 9.38 metric tons. Among the farmed crustaceans, shrimps are considered as one of the major and popular cultured species with high economical value. In 2019, the production of shrimps was estimated as 4.5 million tons.

However, the intensive nature of the aquaculture systems along with the global warming phenomena is a predisposing cause of the diseases of cultured species specially in the scenario of bacterial diseases. Vibriosis caused by the numerous species of *Vibrio* sp. is still one of the major bacterial diseases in shrimp aquaculture systems. Mostly, the distribution of *Vibrio* sp. has been extended recently due to global warming and the intensification of culture systems. Because of that, the amplification of existing pathogenic strains and spreading of novel virulence to commensal bacteria are occurred as most of the *Vibrio* sp. can change their virulence with the acquisition of the genetic material s via horizontal gene transfer.

Numerous scientific research about the different species of *Vibrio* sp. and the occurrence of vibriosis have been done and still ongoing as with the major goal in prevention and control of the disease outbreaks in major shrimp farming countries. The present study is also can be considered as a part of the mentioned research. For the present research, we studied about *Vibrio nigripulchritudo* as one of the emerging causative agents of vibriosis. It was firstly isolated in New Caledonia in 1995 from diseased blue shrimps *Penaeus stylirostris* and here after considered as an etiological agent responsible for the occurrence of summer syndrome in penaeid shrimps. First reported event of mass mortality of Kuruma shrimp *Penaeus japonicus* due to *V. nigripulchritudo* infection in Japan was occurred in 2005.

Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of

*V. nigripulchritudo* infection research. A new revolution has begun on bacterial diseases with the onset of bacterial whole genome sequencing for the discovery of the virulence related genes involved in pathogenicity of respective microbial pathogens. Therefore, the study of genes and their functions, host-pathogen interactions and comparative genomics with other related species would allow a complete understanding on pathogenicity and virulence of *V. nigripulchritudo.*

Intending to acknowledge the pathogenic processes involved in a particular disease and to treat and prevent the disease successfully, the isolation and the identification of the respective etiological agent is highly required. Compared with the other bacterial species, *Vibrio* sp. are easier to isolate from their environment after provisioning of their specific growth factors. For the isolation of the *Vibrio* sp., the media must be supplemented with salt (NaCl) to equivalent to the natural marine environment of *Vibrio* sp. Using both TCBS and HI agar medium supplemented with 2.5% salt, we were able to isolate *V. nigripulchritudo* as pure and healthy colonies after an overnight incubation in the optimum temperature of 25 $^{o}$C.

**In Chapter 2, the identification, and the confirmation of the isolated *V. nigripulchritudo*** is mainly discussed. The isolated bacterial strain from the diseased white leg shrimps *P. vannamei* were identified and confirmed as *V. nigripulchritudo* using the PCR amplification with 16s RNA and hemolysin primers. Specially in the case of amplification with hemolysin primer, the positive results were only obtained for the isolated strain added with hemolysin primer designed for *V. nigripulchritudo* indicating a higher specificity of hemolysin gene for the identification of *Vibrio* related species. The obtained sequence data from Sanger sequencing aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn) showed that all the isolated strains of the bacteria (TUMSAT-V. nig1, TUMSAT-V. nig2, TUMSAT-V. nig3) are having the highest homology with the available reference genomes of *V. nigripulchritudo* in gene bank. Concurrently, results of the challenge tests showed that the isolated strains were pathogenically virulent for Kuruma shrimp *P. japonicus* in both immersion and injection infection indicating that the disease outbreaks due to *V. nigripulchritudo* could be able to cause severe damage to shrimp farming industry. Also, the isolated strain was identified as *V. nigripulchritudo* based on the agglutination test with respect to its antigen binding capability.

The present isolate showed high pathogenicity to Kuruma shrimp, *P. japonicus* in the immersion and injection experimental infections. It indicates that the disease outbreaks due to *V. nigripulchritudo* could be able to cause severe damage to shrimp farming industry. However, the dose specificity, strain specificity of the respective bacteria for different species of penaeid shrimps needed to be further analyzed to get a better insight about the pathogenicity.

**In Chapter 3, the genomic characterization of the isolated strains of *V. nigripulchritudo*** along with the presence of virulence related genes and mobile genetic elements were discussed while focusing the attention of the comparative genome analysis with closely related *V. nigripulchritudo* strains. Compared to the other bacterial pathogens belonging to family Vibrionaceae, the whole genome sequence and annotation of *V. nigripulchritudo* is yet to be done to fulfill the breakthrough of *V. nigripulchritudo* infection research. Therefore, the Illumina and Nanopore whole genome sequencing technology was used to create hybrid genomic assemblies as the long reads provide information on the genome structure while the short reads provide high base-level accuracy. The genome assembly of the isolated strains of *V. nigripulchritudo* has revealed that all the strains are occupied with two chromosomes (chromosome I and chromosome II) with the presence of either one or two plasmids in their genomes.

Numerous virulence related genes are present in either chromosome I or chromosome II. Adherence is found with Mannose-sensitive hemagglutinin and Type IV pilus while antiphagocytosis is observed with capsular polysaccharides. Flagella are used to maintain chemotaxis and motility in the isolated strains. Iron uptake is governed with Vibriobactin, Enterobactin receptors and Heme receptors. For the quorum sensing, auto inducer-1 and cholera auto inducer-2 are found in chromosome I and chromosome II respectively. As bacterial secretion systems for secretion of substances as their virulence factors (mainly of proteins) to invade the host cells, the isolated strains are found with EPS type II secretion system and VAS type VI secretion system. Hemolysin gene is found in the chromosome II of the isolated strain as a potential virulence factor which causes lysis of red blood cells of the host.

Presence of genomic islands, prophages and antibiotic resistance genes have indicated the evidence of the horizontal gene transfer events occurred in the isolated strains *V. nigripulchritudo* with the acquisition new genetic information through HGT, which has a significant role in bacterial evolution and affects pathogenesis. The comparative genomic analysis based on whole-genome proteome data of five *V. nigripulchritudo* genomes (three isolated strains with two reference genomes of *V. nigripulchritudo*) showed that the resulting tree split the *V. nigripulchritudo* strains into three clusters. The higher evolutionary homology is observed in the TUMSAT-V. nig1 and TUMSAT-TG-2018 followed by TUMSAT-V. nig2 and TUMSAT-V. nig3 respectively while the SFn1-2013 isolated from New Caledonia split into a separate cluster.

With these findings of the genome assembly and annotated genomic features related with the virulence factors and mobile genetic elements of the isolated strains of *V. nigripulchritudo* TUMSAT-V. nig1, TUMSAT-V. nig2, and TUMSAT-V. nig3, we can conclude that our study would be important for the prevention and the control of the spread of the vibriosis disease in penaeid shrimps as the present study provides valuable information for understanding the genetic features, pathogenicity, phylogeny, and evolution of *V. nigripulchritudo*.