INSTITUTO POLITÉCNICO NACIONAL

CENTRO DE BIOTECNOLOGÍA GENÓMICA

"CHARACTERIZATION AND GENOME ANALYSES OF *BDELLOVIBRIO* STRAINS ISOLATED FROM DIFFERENT ENVIRONMENTAL SOURCES IN REYNOSA, TAMAULIPAS, MEXICO"

THESIS SUBMITTED

TO OBTAIN THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN BIOTECHNOLOGY

BY

OMOTAYO OPEMIPO OYEDARA

B. Tech (Microbiology), LAUTECH, Ogbomoso, Nigeria

MSc. (Microbiology), OAU, Ile-Ife, Nigeria

Reynosa, Tamaulipas, México **December, 2017**

INSTITUTO POLITÉCNICO NACIONAL

CENTRO DE BIOTECNOLOGÍA GENÓMICA

"CHARACTERIZATION AND GENOME ANALYSES OF *BDELLOVIBRIO* STRAINS ISOLATED FROM DIFFERENT ENVIRONMENTAL SOURCES IN REYNOSA, TAMAULIPAS, MEXICO"

THESIS SUBMITTED

TO OBTAIN THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN BIOTECHNOLOGY

BY

OYEDARA OMOTAYO OPEMIPO

B. Tech (Microbiology), LAUTECH, Ogbomoso, Nigeria

MSc. (Microbiology), OAU, Ile-Ife, Nigeria

Reynosa, Tamaulipas, México **December, 2017**

CERTIFICATION

I hereby certify that the Ph.D. thesis entitled **"Characterization and Genome Analyses of *Bdellovibrio* Strains Isolated from Different Environmental Sources in Reynosa, Tamaulipas, Mexico"** which is submitted by **M.Sc. OMOTAYO OPEMIPO OYEDARA** for the degree of **DOCTOR OF PHILOSOPHY (Ph.D.)** in **BIOTECHNOLOGY** to Instituto Politécnico Nacional (IPN), Mexico is the result of research work done by him in the Laboratorio de Biomedicina Molecular, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Reynosa, Tamaulipas, Mexico under my guidance and supervision during the period of January, 2014 – December, 2017.

I further declare that the result of this work have not been previously submitted either partially or fully for any other degree or fellowship.

Reynosa, Tamaulipas, Mexico

…… ……

Dr. Mario Alberto Rodríguez Pérez **Date and Place**

Supervisor

DECLARATION

I hereby declare that the Ph.D. thesis entitled **"Characterization and Genome Analyses of *Bdellovibrio* Strains Isolated from Different Environmental Sources in Reynosa, Tamaulipas, Mexico"** which is submitted herewith for the degree of **DOCTOR OF PHILOSOPHY (Ph.D.)** in **BIOTECHNOLOGY** to Instituto Politécnico Nacional (IPN), Mexico is the result of research work done in the Laboratorio de Biomedicina Molecular, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Reynosa, Tamaulipas, Mexico under the supervision of **Dr**. **MARIO ALBERTO RODRÍGUEZ PÉREZ** during the period of January, 2014 – December, 2017.

I further declare that the result of this work have not been previously submitted either partially or fully for any other degree or fellowship.

Reynosa, Tamaulipas, Mexico

……..…… ……..……..

Omotayo Opemipo Oyedara (M.Sc.) **Date and Place**

DEDICATION

I dedicate this work to my family, friends and all who supported and contributed to the success of this Ph.D. work. And also to all scientists and individuals who genuinely work tirelessly day and night to sustain the existence of all beautiful life forms created by only one supreme supernatural being, Almighty God.

Genius is only the power of making continuous efforts
The line between failure and success is so fine that we scarcely know when we pass it
So fine that we are often on the line and do not know it
How many a man has thrown up his hands at a time when a Little more effort and
A Little more patience would have achieved success
Elbert Hubbard

ACKNOWLEDGEMENT

I would like to express my gratitude to my thesis director, Dr. Mario Alberto Rodríguez Pérez for his support and the opportunity to grow under his tutelage during the course of this research work. I also want to appreciate members of my thesis committee – Dr. X. Guo, Dr. Victor R. M. Medina, Dr. Juan M. G. Prieto, Dra. Ninfa M. R. Garcia, and Dra. Patricia L. Corona for your constructive criticism.

I want to acknowledge Consejo Nacional de Ciencia y Tecnologia (CONACyT), Secretaria de Investigacion y Posgrado del IPN (SIP), and Programa Institutional de Formacion de Investigadores - PIFI-IPN (BEIFI) for providing grants and scholarship for this Project. I will also like to acknowledge the Management of Osun State University for granting me study leave.

I would like to appreciate the significant contributions of the following people to the success of this project: Dr. Virgilio B. Garcia and Dra Ana Veronica M. Vasquez for providing some bacterial strains, Dr. Alberto Mendoza H. and his laboratory team (Susana Fernandez, Antoinette Cruz and Eyra O. de Guzman) for assisting with the southern blotting technique. I want to acknowledge Dra. Guadalupe Rojas and Dr. Menchaca of UANL, San Nicolas, Mexico for assisting with bacterial strains and the atomic force microscopy. I wish to thank Prof. Jurkevitch Edouard and Rita Petrenko of Department of Plant Pathology and Microbiology, the Hebrew University of Jerusalem, Israel for magnanimously providing us with *Bdellovibrio bacteriovorus* HD100. I appreciate Dr. Aldo S. Cabrera for accepting me to carry out a research stay at INECOL, Xalapa during the bioinformatic aspect of this work. I want to extend my gratitude to Dr. Erick J de Luna Santillana and Omar O. Rodriguez for your immense contributions and brotherly assistance. Thank you, Francisco Juahey and Juan-Carlos Antunez for your assistance.

I want to appreciate Prof. Adeleke M. A. who gave the idea to study my Ph.D. in this reputable institution – Centro de Biotecnologia Genomica, Instituto Politecnico Nacional (CBG-IPN), Reynosa, Mexico. My gratitude also goes to my M.Sc. supervisor, Prof. A. O. Shittu for your significant contributions to my academic career. A huge thank you to all the staff of CBG-IPN for

their academic assistance and kindness during my stay in the institution. I also appreciate the Head of Postgraduate studies, CBG, QFB. Clara P. D. Villareal for her assistance.

I appreciate members and colleagues in the Laboratorio de Biomedicina Molecular during my stay in the Lab (Lihua, Alfonso, Fanny, Cristina, Edgar, Mary, Melina, Rogelio and Mariel), and those that worked directly with me in this Project (Rafael, Litany and Sofia) for their constructive scienific discussions and happy moments together. To all my wonderful Mexican (Israel Vergara, Gaston, Eric, Blanca, Daryana, Deysi, Daryana, Aureliano, Sarahi, Paco and Ulises) Colombian (Viviana Caesar, Jazmin, and Andrea), Pakistan (Kashif) and Haitian (D. Nelson and Mandee), colleagues and friends, I say a big thank you. Thank you, Xochitl. P. for being there for me. I appreciate all my “paisanos” in Reynosa (Mr. Eziekel A. Onaleye (Bishop) and his family, Temidayo, Biyi, Osagie, Ibraheem and Bimbo) for times spent together as one family. My gratitude also goes to Maestro Alex and Maestra Isabel, Dra. Elma L. Salazar, Maestra Socorro Ramirez, the families of Sra. Lira Vazquez, Sra. Vargas Magaña, Karla Fuentes Rodriguez, and Doña Rosa for their kind gestures towards me and my family.

It is important to sincerely and specially express my profound gratitude to my dear wife, Engr. Abiola A. Oyedara and lovely daughters, Oluwademilade and Oluwadunsin for their absolute support and understanding during my Ph.D. program. I want to appreciate the families of Arch. and Dr. (Mrs) Adeyemi S. O., Hon. Adeyemi Rasaq, Engr. Afolabi O. Oyedara, Pharm. Toyosi Akanbi, Mrs. Funmilola Bamkefa, Tolulope Gregory Oyedara, Chief G. Fadahunsi, Mr Bayo Dahunsi, Mrs Olaniyan and Dr Akinde S. B. for their prayers, assistance and financial supports towards the success of this Ph.D. program. I specially appreciate my source of existence, Engr Adedokun T. and Omolola A. Oyedara because by the special grace of God, this journey of my life started with you.

And to all those that have contributed to this great achievement, I say “Muchisima Gracias”

Finally, to almighty God, who blessed me with life, good health, wisdom and understanding to complete this important stage of my career. What can I do without You Lord, the potter of my life? Absolutely Nothing!

TABLE OF CONTENT

LIST OF ABBREVIATIONS

%	Percentage
µL	Microliters
µm	Micrometer
AAI	Amino acid Identity
AFM	Atomic Force Microscopy
ANI	Average nucleotide Identity
ARDRA	Amplified ribosomal ribonucleic acid restriction analysis
ATCC	American Type Culture Collection
BALOs	Bdellovibrio and Like Organisms
BLAST	Basic Local Alignment Search Tool
bp	Base pair
$CaCl_2$	Calcium Chloride
CDBB	Colección Nacional de Cepas Microbianas y Cultivos Celulares
CDS	Coding sequences
CFU	Colony forming unit
CINVESTAV	Centro de Investigación y de Estudios Avanzados
d-BALOs	Deltaproteobacteria-Bdellovibrio and Like Organisms
DDH	Deoxyribonucleotide acid to deoxyribonucleotide acid Hybridization
DNA	Deoxyribonucleic acid
DNB	Dilute Nutrient Broth
dNTP	Deoxyribonucleotide diphosphate
g	Grams
g/L	Grams per Litre
GC/G+C	Guanine cytosine content
gDNA	Genomic DNA
h	Hour

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hit	Host interaction locus
IPN	Instituto Politecnico Nacional
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobases
LB	Luria Bertani
mμ	Millimicron
Mb	Megabases
$MgCl_2$	Magnesium chloride
MHA	Mueller Hinton Agar
min	Minutes
mL	Milliliter
mm	Millimeter
N	North coordinate
NCBI	National Center for Biotechnology Information
ncRNA	Non coding ribonucleic acid
ng/μL	Nanograms per microliter
NGS	Next generation Sequencing
nM	Nanomolar
NNNN	Unknown Nucleotide Sequences
^{o}C	Centigrades
ORF	Open reading frames
PCR	Polymerase Chain Reaction
PD	Prey dependent
PFU	Plaque Forming Unit
pH	Hydrogen potential
PI	Prey-independent
qPCR	Quantitative polymerase chain reaction

RAST	Rapid Annotation Using Subsystem Technology
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
sp.	Species (singular)
spp.	Species (plural)
tRNA	Transfer ribonucleic acid
UANL	Universidad Autonomas de Nuevo Leon
VFDB	Virulence factor Database
W	West coordinate
w/v	Weight per volume
x g	times gravity
X-gal	X-galactosidase (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)
YP	Yeast peptone
δ	Delta

LIST OF FIGURES

LIST OF TABLES

RESUMEN

Las especies del género *Bdellovibrio* corresponde a pequeñas bacterias gram negativas de 0.2-0.5μm × 0.5-2.5μm, uniflageladas, móviles, atacan e hidrolizan los componentes celulares de otras bacterias gram-negativas utilizando los nutrientes derivados del proceso de digestión para el crecimiento y la reproducción. Las características depredadoras de *Bdellovibrio* spp. las convierte en una herramienta de biocontrol útil en áreas como: medicina, acuicultura, ganadería, industria alimenticia y horticultura. Sin embargo, a pesar de las numerosas investigaciones sobre esta bacteria benéfica, hay una escasez de información sobre *Bdellovibrio* spp en México. Por lo tanto, esta investigación se enfocó en el aislamiento, caracterización y análisis del genoma de cepas de *Bdellovibrio* provenientes de suelos y aguas residuales del municipio de Reynosa, Tamaulipas, México para entender el estilo de vida de *Bdellovibrio* spp y emplearla como agente de biocontrol. Se aislaron cinco cepas de *Bdellovibrio* spp, presentes en muestras de suelo, mediante la técnica de cultivo dependiente y empleando a tres miembros de la familia Enterobacteriaceae (*Klebsiella* sp., *Salmonella* sp. y *Citrobacter freundii* ATCC 8090) como presa. Las cepas de *Bdellovibrio* aisladas son gram-negativas, bacteriolíticas, formadoras de placas y altamente móviles. Las cepas de *Bdellovibrio* se identificaron y confirmaron mediante microscopía, amplificación por PCR del locus '*hit'* y con secuenciación del gen 16S rRNA. Se determinó que eran diferentes cepas basadas el locus '*hit'* y con el análisis de amplitud de presas. Toda esta información confirma las investigaciones anteriores que describen el comportamiento heterogéneo de las poblaciones de *Bdellovibrio* spp, por lo que es necesario identificar la diversidad de las cepas.

El segundo objetivo de este estudio, se enfocó en aislar el fenotipo con crecimiento independiente de presa, "*host independent*", de las cepas de *Bdellovibrio* utilizando tres métodos diferentes descritos en la literatura, sin embargo, no se aislaron con éxito. Así, las colonias bacterianas aisladas con estos métodos se asemejaban fenotípicamente a las cepas de *Bdellovibrio* con crecimiento independiente descritas en la literatura, pero resultaron negativas para la caracterización y confirmación por PCR del gen 16S rRNA y del locus '*hit'*. Este resultado interrumpió el estudio de las características bioquímicas de estas cepas.

A través de la secuenciación del genoma completo y los estudios comparativos del mismo, se puede proporcionar información acerca de la naturaleza depredadora de *Bdellovibrio* spp, como su adaptación ecológica y, al mismo tiempo, ayudar con éxito en su uso como control biológico contra patógenos. Se secuenció el genoma de dos aislados independientes de *Bdellovibrio* spp (SKB1291214 y SSB218315) mediante secuenciación de siguiente generación tipo Illumina. El genoma completo de *B. bacteriovorus* SSB218315 arrojó un tamaño de genoma de 3,769,537 pb y un contenido GC de 50.5%, mientras que el borrador final del genoma de *Bdellovibrio* spp. SKB1291214 tiene un tamaño de 3,724,490 pb, conformado por 20 contigs y un contenido GC del 44.8%. Los factores genéticos encontrados que pueden ayudar en la depredación de *Bdellovibrio* spp. se incluyen: el flagelo, pili tipo IV, quimiotaxis, factores asociados a toxinas y un buen número de enzimas degradativas. El análisis comparativo de genomas basado en la identidad de aminoácidos (AAI) y secuencia del gen 16S rRNA reveló la relación genómica entre las cepas del estudio y otros 7 genomas depositados en la base de datos NCBI. Las cepas de *Bdellovibrio* aisladas mostraron diferencias basadas en la secuencia del gen 16S rRNA con *Bdellovibrio* spp compartiendo una similitud del 99% con una cepa no cultivable de *Bdellovibrio* sp 12L 106 (distancia entre parejas de 0,008) y una identidad de 95-97% (distancia entre parejas de 0,043) con otras cepas cultivables de *Bdellovibrio* spp, incluyendo la cepa SSB218315. *Bdellovibrio* sp. SKB1291214 presentó un AAI bajo con otras cepas (63.7 – 67.68 %) mientras que *B. bacteriovorus* SSB218315 compartió una alta identidad de aminoácidos (95 %) con cepas de *B. bacteriovorus* HD100, Tiberius y 109J. Por lo tanto, tomando en cuenta el porcentaje de contenido de GC, el patrón de agrupación de árboles filogenéticos y el valor de AAI, la cepa SKB1291214 podría ser una nueva especie. Además, *Bdellovibrio* sp. SKB1291214 también poseyó 30 grupos de genes únicos, algunos de los cuales podrían haber sido adquiridos a través de la transferencia horizontal de genes. Además, las variaciones en la secuencia del gen *Bd0108*, el cual está relacionado a la adherencia, ataque e invasión de la presa, como el establecimiento del fenotipo de crecimiento independiente de presa observados en *Bdellovibrio* sp. SKB1291214 puede ser un factor responsable de la reducción de su capacidad depredadora en comparación con la cepa

SSB218315. Estos estudios del genoma proporcionan información que puede ayudar a la aplicación de Bdellovibrio spp. como un agente de control biológico.

ABSTRACT

Bdellovibrio spp are small (0.2-0.5µm × 0.5-2.5µm), uniflagellated, motile Gram negative bacteria that attacks and hydrolyzes cellular constituents of other Gram-negative bacteria. They utilize the derived nutrients from the digestion process for growth and reproduction. The predatory characteristics of *Bdellovibrio* sp makes them suitable as a biocontrol agent against different bacterial pathogens in different fields including medicine, aquaculture, animal husbandry, food industry, and horticulture. However, despite numerous publications and researches on this beneficial bacteria, there has been a paucity of information on *Bdellovibrio* spp in Mexico. In an effort to understand the predatory lifestyle, and apply *Bdellovibrio* spp. as a biocontrol agent, we focus this research on the isolation, characterization and whole-genome analyses of *Bdellovibrio* strains isolated from soil and sewage in Reynosa, Tamaulipas, Mexico. In this study, five strains of *Bdellovibrio* were isolated from soil samples using the culture-dependent technique and three members of the family Enterobacteriaceae (*Klebsiella* sp., *Salmonella* sp., and *Citrobacter freundii* ATCC 8090) as prey. The *Bdellovibrio* strains were bacteriolytic, plaque-forming, and highly motile gram-negative bacteria. We identified and confirmed the *Bdellovibrio* strains using microscopy, PCR amplification, and sequencing of the 16S rRNA gene. They were observed to be different strains based on *hit* locus and prey range analyses. Here, the first report on *Bdellovibrio* strains isolated from soil in Mexico corroborates earlier report indicating that populations of *Bdellovibrio* spp are heterogeneous thereby the need to identify the various strains.

In the second objective of this study, several attempts to culture the prey-independent phenotypes of the isolated *Bdellovibrio* strains using three different methods earlier described in literature was not successful. However, bacterial colonies isolated with this methods phenotypically resembled the prey-independent *Bdellovibrio* strains described in different publications but negative for characterization and confirmation based on PCR amplification of the 16S rRNA gene and *hit* locus. This result prevented us from studying the biochemical characteristics of these strains.

An insight into the predatory nature of *Bdellovibrio* spp. through whole genome sequencing and comparative genome studies can provide information on their ecological adaptation and assist their successful application as a biocontrol agent against pathogens. We sequenced the genomes of two different *Bdellovibrio* spp. SKB1291214 and SSB218315 isolated from soil using Illumina Next Generation Sequencing technology. The complete genome of *B. bacteriovorus* SSB218315 yielded a genome size of 3,769,537 bp and GC content of 50.5 % while final draft genome of *Bdellovibrio* sp. SKB1291214 has a size of 3,724,490 bp, 20 contigs and GC content of 44.8 %. The factors found in the genomes that can aid predation in the *Bdellovibrio* spp. include flagellar, Type IV pilus, chemotaxis, toxin associated factors and quite numbers of degradative enzymes. Comparative genome analysis based on amino acid identity and 16S rRNA gene sequences revealed information on the genomic relatedness between the study strains and 7 other sequenced genomes retrieved from the NCBI database. The *Bdellovibrio* strains exhibited differences based on the 16S rRNA sequence with *Bdellovibrio* sp. SKB1291214 sharing 100 % similarity with an uncultured *Bdellovibrio* sp clone 12L 106 (a pairwise distance of 0.008) and 95 - 97 % (a pairwise distance of 0.043) identity with other culturable terrestrial *Bdellovibrio* spp. including strain SSB218315. *Bdellovibrio* sp. SKB1291214 shared low AAI with other strains (63.7 – 67.68 %) while *B. bacteriovorus* SSB218315 shared high amino acid identity (95 %) with *B. bacteriovorus* strains HD100, Tiberius and 109J. Therefore, considering the percentage GC content, phylogenetic tree clustering pattern, and AAI value, strain SKB1291214 could be a novel species. Furthermore, *Bdellovibrio* sp. SKB1291214 also possessed 30 unique gene clusters, some of which might have been acquired through horizontal gene transfer. In addition, variations in the sequence of a *Bd0108* gene, attributed to prey attachment, invasion, and development of prey-independent *Bdellovibrio* phenotypes observed in *Bdellovibrio* sp. SKB1291214 may be a contributory factor responsible for the reduction in its predatory capacity compared to strain SSB218315. This genome study provide information that can aid the successful application of *Bdellovibrio* spp. as a biocontrol agent.

CHAPTER ONE

1.1. GENERAL INTRODUCTION

Predation is one of the microbial interactions that can occur in the complex terrestrial and aquatic environment. Bacteria can be preyed upon by protists, bacteriophage or prokaryotes. *Bdellovibrio* and Like Organisms (BALOs) viz. genus *Bdellovibrio*, *Bacteriovorax*, *Peredibacter*, *Halobacteriovorax* and *Micavibrio* are a group of obligate predatory bacteria that prey upon gram-negative bacteria for nutrients and reproduction. *Bdellovibrio* spp., are members of the BALOs representing one of the most studied predatory bacteria. They are small (0.2–0.5 μm × 0.5–2.5 μm), uniflagellated motile gram-negative bacteria that attack and hydrolyze cellular constituents of other gram-negative bacteria, utilizing the derived nutrients for growth and reproduction.

Bdellovibrio spp. are ubiquitous in nature and they have been isolated from different sources including plant rhizospheres, freshwater, soil, and gastrointestinal tract of animals. Based on the mechanism of predation, the genus *Bdellovibrio* is grouped into two species namely *B. bacteriovorus* and *B. exovorus*. The former has the ability to invade the periplasmic space of its prey while the latter only attach to the external surface of the prey (epibiotic) to derive nutrients. Members of the Genus *Bdellovibrio* have been reported to be diverse in nature with heterogeneous groups inhabiting same ecological niche, and some members exhibiting unique features such as the development of dormant structure called Bdellocyst which aid survival during unfavourable condition as observed in *Bdellovibrio* sp. strain W.

Several publications have indicated *Bdellovibrio* spp. as a better alternative to treat infections caused by multidrug-resistant bacteria. It has also been suggested as a biocontrol agent in aquaculture and animal husbandry. *Bdellovibrio* is also capable of degrading or inhibiting biofilms produced by both gram-positive and -negative bacteria. Furthermore, it can be used as a novel, biological lytic agent for the inexpensive, industrial-scale recovery of intracellular products such as polyhydroxyalkanoates from different Gram-negative prey cultures. Another important feature that

qualifies *Bdellovibrio* as a successful biocontrol agent is that it has been shown to be non-pathogenic to mammals or humans.

Though described as an obligate intracellular predator, *Bdellovibrio* strains that are capable of growing in the absence of prey as well as on nutrient-rich media mostly referred to as host-independent *Bdellovibrio* strains have been isolated using current laboratory protocols. In the study, we prefer to use the word “prey” instead of “host”. The molecular derivation of prey-independent (PI) *Bdellovibrio* strains has been linked with a mutation in a genetic *hit* (host interaction) locus coding for proteins that play an active role in the attachment and invasion of *Bdellovibrio* into its prey.

Bdellovibrio bacteriovorus sequenced genome and proteome analysis by Schuster *et al*., 2004 and Pan *et al*., 2011 revealed that despite the small size *of B. bacteriovorus*, it possesses large genome size of approximately 3.8 Mb coding for numerous hydrolytic enzymes that enhance its predatory activities. And despite easy access to prey genetic material, only a few lateral gene transfer has been reported in *B. bacteriovorus.*

With *Bdellovibrio* spp. possessing the aforementioned advantageous qualities, it is pertinent to study their predatory lifestyle for the ultimate purpose of applying them as a biocontrol agent against pathogens. However, there is limited information on this useful bacteria in Mexico. Therefore, in an effort to further understand the predatory lifestyle and achieve successful application of *Bdellovibrio* spp. this study focuses on the isolation, characterization and whole-genome analyses of *Bdellovibrio* strains isolated from different environmental sources in Reynosa, Tamaulipas, Mexico.

1.2. JUSTIFICATIONS

The justifications for this study are highlighted below:

1. The environment as a community of diverse microorganisms will be an excellent source to isolate *Bdellovibrio* strains that can be used in controlling pathogenic bacteria including the drug-resistant ones.
2. Members of Bdellovibrionaceae have been reported to show great phylogenetic diversity. It is therefore important to characterize the different strains of *Bdellovibrio* that can be isolated from different environmental sources.
3. *Bdellovibrio bacteriovorus* has been reported to experience only a few horizontal gene transfer. This shows that there is a relative stability in the genome of *B. bacteriovorus*, and this can be an advantage for its biocontrol application. However, it is important to ascertain these characteristics in different strains of *Bdellovibrio*.
4. There is a paucity of data on *Bdellovibrio* strains in Mexico. This is the first report on *Bdellovibrio* in Mexico and the information obtain in this study can serve as baseline study on *Bdellovibrio* strains in Mexico.

1.3. HYPOTHESIS

The hypothesis of this work is *Bdellovibrio* strains capable of preying upon a wide range of Gram-negative bacteria can be isolated from environmental sources in Reynosa, Tamaulipas, Mexico with expectation that these strains will exhibit notable genomic variations when compared with other reported *Bdellovibrio* strains.

1.4. GENERAL OBJECTIVE

The general objective of this work is to isolate, characterize and carry out whole genome analyses of *Bdellovibrio* strains from different environmental sources in Reynosa, Tamaulipas, Mexico.

1.5 SPECIFIC OBJECTIVES

The specific objectives include:

1. To isolate and carry out the phenotypic and molecular characterization of prey-dependent *Bdellovibrio* strains from different environmental sources in Reynosa, Tamaulipas, Mexico.
2. To carry out the phenotypic and molecular characterization of prey-independent *Bdellovibrio* phenotypes that will be derived from the successfully isolated prey-dependent *Bdellovibrio* strains from the objective (1) above.
3. To carry out whole genome sequencing and analyses of selected isolated *Bdellovibrio* strains.

CHAPTER TWO

Objective 1. Isolation, Phenotypic and Molecular Characterization of Prey-dependent *Bdellovibrio* Strains from Different Environmental Sources in Reynosa, Tamaulipas, Mexico.

2.1. INTRODUCTION AND LITERATURE REVIEW

2.1.1 BACTERIAL PREDATION

Prokaryotic predation is an old important phenomenon dated to the time of prokaryotes existence, about 3.5 billion years ago. It has been described as means of survival among prokaryotes before the emergence of first eukaryotes about 1 to 1.5 billion years ago. Moreover, predation among prokaryotes has even been suggested to play an evolutionary role in the development of eukaryotic organelles such as mitochondria (Davidov and Jurkevtch, 2009).

In the natural environment, prokaryotic predatory bacteria have been described to use different strategies to attack their prey (Table 2.1). Some predatory bacteria can attack in a group, referred to as Wolfpack (Figure 2. 1). These bacteria do not necessarily need to make contact with their prey, rather they excrete several hydrolytic enzymes into the environment to digest their prey. The resulting products of digestion are then assimilated and utilised by the predator for survival. This kind of predatory strategy has been reported in *Myxococcus xanthus*, a facultative predatory bacterium (Muñoz-Dorado *et al*., 2016). Some predatory bacteria such as Vampirococcus and *Bdellovibrio exovorus* exhibit a predatory strategy known as epibiotic (Figure 2. 2). In this type of strategy, the predatory bacteria do not penetrate the prey but attached to the external surface of the prey and excrete hydrolytic enzymes that will digest the prey, releasing the cellular content for predator assimilation (Guerrero *et al*., 1986; Koval *et al*., 2013). Predatory bacteria such as Daptobacter directly invade the cytoplasm of their prey, digest the cytoplasmic contents and use the resulting product for growth and reproduction (Guerrero *et al*., 1986) (Figure 2. 3). Lastly, another strategy that can be used by predatory bacteria is the invasion of the periplasmic space (Guerrero *et al*., 1986; Strauch *et al*., 2007) (Figure 2. 4). *Bdellovibrio* and like organisms (BALOs) most especially *Bdellovibrio* spp. are examples of predators that use this strategy, and they are the main focus in this study.

Table 2. 1. Comparative features of predatory prokaryotes (Olanya and Lakshman, 2015).

	Distribution/ ecology	Morphology features*	Gram reaction	Aerobic reaction	Motility	Predator to prey location	Prey hosts	Multiplication	Taxonomy/ classification	Type of predation
Bdellovibrio/ Bacteriovorax	Soil Seawater and fresh water	Curved rods 0.35×1.2µm	G -ve	Strictly aerobic	Single, polar sheathed flagellum	Periplasmic space	G –ve bacteria	Bdelloplasts	Delta proteobacteria	Obligate
Daptobacter	Fresh water	Rod shape, 0.5×1.5µm	G -ve	Facultative anaerobic	Single, polar, unsheathed flagellum	Endobiotic (within the Host prey)	Phototropic microbes	Binary fission	Delta proteobacteria	Endobiotic
Myxobacteria	Aquatic / seawater soil	Ovoid / spheres	G -ve	Partially aerobic	Gliding motion	Encircle prey (wolf pack)	G –ve	Myxospores/ Multi-cellular Fruiting bodies	Delta proteobateria	Facultative
Micavibrio	Waste water	Rod, curved 0.5-1.5µm	G -ve	Facultative anerobic	Motile, Polar flagellum	Epibiotic	G -ve	Binary fission	Alpha proteobacteria	Obligate
Lysobacter	Soil Fresh water	Rod 0.6-2.5µm	G -ve	aerobic	Gliding fimbriae	Wolf pack	G -ve	---	Gamma proteobacteria	Facultative
Vampiricoccus	Fresh water and sulfurous water	Ovoid, 0.5×0.7µm	G -ve	Anaerobic	Non-motile	Epibiotic (attached to cell wall of prey)	Phototrophic microbes	Binary fission	Not determined (related to Delta proteobacteria)	Epibiotic

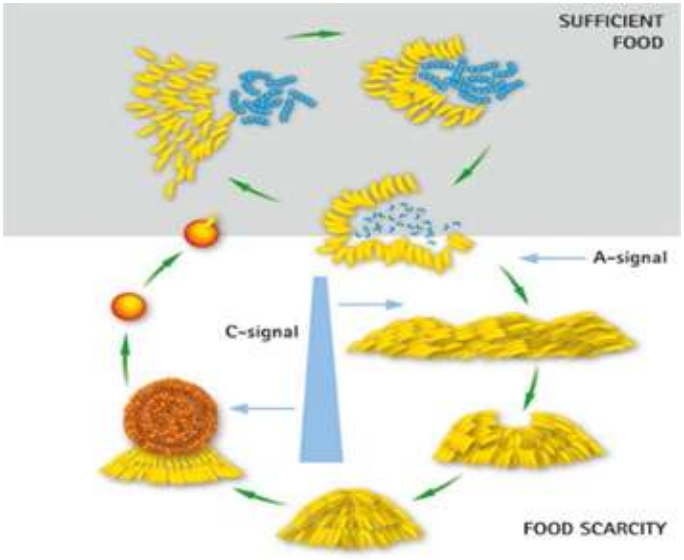


Figure 2. 1. *Myxococcus xanthus* using wolfpack strategy to derive nutrient from its prey.

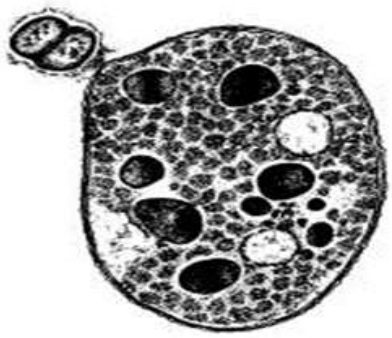


Figure 2. 2. Description of epibiotic mode of attack as observed in *Bdellovibrio exovorus* and Vampirococcus (Guerrero *et al*., 1986)

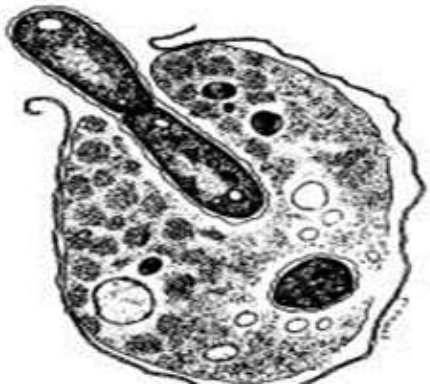


Figure 2. 3. Description of Direct cytoplasmic invasion by Daptobacter (Guerrero *et al*., 1986)

Figure 2. 4. Description of Intraperiplasmic invasion by *Bdellovibrio bacteriovorus* (Guerrero *et al.,* 1986)

2.1.2. *BDELLOVIBRIO* AND LIKE ORGANISMS (BALOs)

Louis Pasteur during a lecture at Université de Lille in December 7, 1854, said "...*Dans les champs de l'observation le hasard ne favorise que les esprits prepares*" (In the fields of observation, chance only favors the prepared mind). The above statement described the kind of diligence and keen observation power demonstrated by Stolp and Petzold, which eventually led to the accidental discovery of the fastest small bacterial "hunters" known as *Bdellovibrio*. Since the discovery of *Bdellovibrio*, research works have been launched to explore the fascinating world of *Bdellovibrio* and other related predatory bacterial species collectively known as *Bdellovibrio* and like Organisms (BALOs).

Bdellovibrio spp. and other related predatory bacteria including *Bacteriovorax* spp, *Peredibacter* spp, *Halobacteriovorax* spp, and *Micavibrio* spp are collectively referred to as *Bdellovibrio* and like Organisms. They are highly motile, uniflagellate, Gram-negative, obligate predatory bacteria that have been described to prey primarily upon other Gram-negative bacteria. The term BALOs was originally ascribed to a group of predatory bacteria that belong to the class delta-proteobacteria. However, *Micavibrio* spp., obligate Gram-negative predatory bacteria that belong to the class alpha-proteobacteria has been reported (Lambina *et al*., 1982) and affliated with the BALOs (Woese 1987). Members of BALOs have been reported to use different types of strategies to attack their prey. For example, *Bdellovibrio exovorus* and *Micavibrio* spp. have been described to employ the epibiotic strategy to attack their prey (Guerrero *et al*., 1986; Koval *et al*., 2013) while other BALOs penetrate the periplasm of their prey to initiate prey digestion (Guerrero *et al*., 1986; Strauch *et al*., 2007).

BALOs occupy several natural ecological habitats. They have isolated from soil (Stolp and Starr, 1963), rhizosphere (Jurkevitch *et al*., 2000), waterbodies – freshwater, marine, brackish water, run-off, and sewage and treatment water plant (Williams *et al*., 1995). They have also been found in human and animal faeces (Schwudke *et al*., 2001), and recently detected as part of the lung microbiome of Cystic fibrosis patients (de Dios Caballero *et al*., 2017). The presence of BALOs in extreme environments such as rocks contaminated with crude oil (C12-11), groundwater

contaminated with high levels of nitric acid bearing uranium waste (300A-H04), hot spring travertine depositions at 55 ^{o}C, deep-sea hydrothermal sites, methanotrophic communities, and arctic marine sediments have also been reported (Jurkevitch and Davidov, 2006).

The taxonomy and classification of BALOs have been dynamic over the years. The delta-BALOs were initially grouped under a single genus, *Bdellovibrio* for several years. However, reports have shown that they are different based on GC content, salt tolerance, host range, fatty acid tolerance, serological reactions, DNA-DNA hybridization, and molecular analysis of the 16S rRNA gene. Based on these differences, d-BALOs were then separated into different genus namely *Bdellovibrio*, *Bacteriovorax, Peredibacter* and *Halobacteriovorax* (Seidler *et al*., 1972; Kramer and Westergaard 1977; Hespell *et al*., 1984). All d-BALOs belong to the order Bdellovibrionales. However, the heterogeneity and phylogenetic diversity observed among the d-BALOs led to their reclassification into two families, Bdellovibrionaceae and Bacteriovoraceae. The phylogenetic distance between Bdellovibrionaceae and Bacteriovoraceae is very large (>20 %) as reported by Davidov and Jurkevitch (2004). However, the genus Bdellovibrionaceae are ancestral to Bacteriovoraceae because 16S rRNA gene secondary structure analysis showed that they contain motifs atypical of the delta-proteobacteria (Schwudke *et al*., 2001; Davidov and Jurkevitch 2004).

In contrast to other bacterial or eukaryotic parasites such as bacteriophages and *chlamydia*, delta-BALOs have large genome sizes (Jurkevitch and Davidov, 2006). This is not unexpected because BALOs produce a number of enzymes that they use in prey lysis. Research on BALOs and other predatory bacteria are on the research focus currently because they have potentials as agents of biocontrol in several fields such as medicine, agriculture and food industries.

2.1.3. TAXONOMICAL EVENTS OF THE BALOs: The Genus *Bdellovibrio*, *Bacteriovorax*, *Peredibacter* and *Halobacteriovorax*

The Genus *Bdellovibrio* was first classified into the family Spirillaceae; spiral and curved bacteria (Burnham and Robinson, 1974). Characterization of Bdellovibrios using conventional taxonomic tools is usually difficult because they are cultured in the presence of a prey bacteria. However, several publications have shown that the genus *Bdellovibrio* are diverse based on different characteristics such as G-C ratios, fatty-acid profiles, membrane protein compositions, antigenic variations, prey ranges, DNA-DNA hybridization, ribotyping and partial sequencing of the 16S rDNA (Guether *et al*., 1993; Kramer and Westergaard, 1977; Marbach *et al*., 1975; Park and Mahadevan, 1988;; Seidler *et al*., 1969, 1972; Severin *et al*., 1981; Sutton and Besant, 1994; Taylor *et al*., 1974; Baer *et al*., 2000; Jurkevitch *et al*., 2000). Based on DNA composition, nucleic acid homology, enzymes migration rate and biochemical activities, *Bdellovibrio starrii* (typed culture; Bd.A3.12) and *Bdellovibrio stolpii* (typed culture; strain Bd. UKi2) were established in addition to *Bdellovibrio bacteriovorus* (Stolp and Starr, 1963; Seidler *et al*., 1972) (Table 2.2). Though *B. stolpii* and *B. starii* are close based on the G + C mol %, they are different based on their genetic make-up (Seidler *et al*., 1972).

Table 2. 2. The species of the genus *Bdellovibrio* (Starr *et al*., 2013)

Species	Trait		
	DNA base ratio (G+C mol%)	Catalase	Protease activity
Bdellovibrio bacteriovorus	50.4	+	low
Bdellovibrio stolpii	42.0	+	high
Bdellovibrio starrii	43.5	−	moderate

The taxonomy studies carried out by Torrella *et al*. (1978) on different strains of *Bdellovibrio* showed a uniformity in their range of genome sizes but variations in the polynucleotide sequences. They reported that *Bdellovibrio* W (43.7% *G* + C), *Bdellovibrio* 3294 (37.4% *G* + C), and *B. stolpii* UKi2 (41.8% *G* + C) shared a common 30% of their genome while *Bdellovibrio stolpii* UKi2, *B. starrii* A3.12 (43.5% G + C), and *Bdellovibrio* W have in common at least 16% of their DNA

polynucleotide sequences. DNA sequence comparison between *Bdellovibrio* 3294 and *B. starii* A3.12 showed that they are distinctly different, having little or no detectable sequences in common. Furthermore, cytochrome spectrum was shown not to be an excellent tool for *Bdellovibrio* taxonomy because all the *Bdellovibrio* strains considered have common cytochrome spectrum. The possibility to sequence and use ribosomal DNA (rDNA) as strong evolutionary tool for determination of phylogenetic relationships in organisms (Weisberg *et al*., 1991) helped further research conducted by Baer *et al*. (2000) in revealing the dynamics in the taxonomy of *Bdellovibrio*. In their study, Baer *et al*. (2000) compared the complete 16S rDNA sequence, and use DNA-DNA hybridization to determine the phylogenetic relatedness that existed between three *Bdellovibrio* spp isolated from soil; *B. bacteriovorus* 100^T, *B. stolpii* UKi2^T and *B. starrii* A3.12^T (Figure 2. 5, Table 2. 3.). Their results showed that the 16S rDNA sequences of *B. bacteriovorus* 100^T and *B. stolpii* UKi2^T shared similarity of 81.7 % while the similarity between *B. bacteriovorus* $100^T$and *B. starrii* A3.12^T was 81.2 %. They further showed that *B. stolpii* UKi2^{T}and *B. starrii* A3.12^{T}are closer sharing 16S rDNA sequence similarity of 90.0 %. The result of the 16S rDNA comparison and the low DNA-DNA hybridization values among the three species prompted Baer *et al*. (2000) to propose the reclassification of *B. stolpii* UKi2^{T}and *B. starrii* A3.12^T into the new genus *Bacteriovorax* gen. nov as *Bacteriovorax stolpii* comb nov. and *Bacteriovorax starrii* comb nov. respectively.

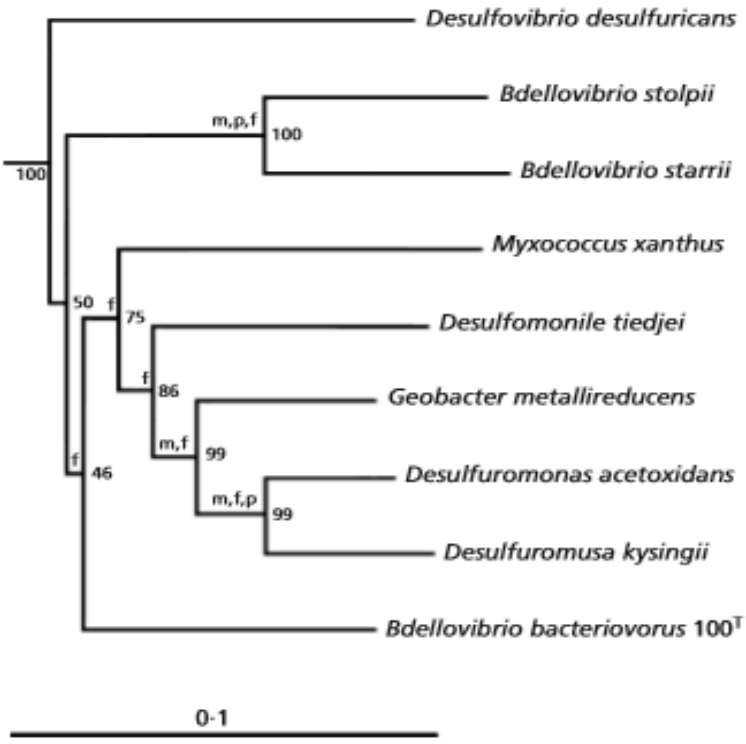


Figure 2. 5**.** Phylogenetic tree showing relationship between *B. bacteriovorus*, *B. stolpii*, and *B.starrii .* Neighbour-joining tree. Phylogenetic analysis was based on 16S rRNA gene sequences (1236 bp) of *Bdellovibrio* sp. and closely related genera. m, f and p indicate branches that were also found using the FASTDNAML, Fitch–Margoliash and maximum-parsimony algorithms, respectively. The numbers at the nodes represent percentages indicating the level of bootstrap support based on a neighbour-joining analysis of 1000 resampled data sets. The tree was generated using *Pasteurella aerogenes* P-172-71T (M75048) and *Xanthomonas phasedi* (C. R. Woese, unpublished results) as outgroups (Baer *et al*., 2000).

Table 2. 3. DNA–DNA hybridization values between different *Bdellovibrio* strains. DNA–DNA hybridization values were determined using a direct binding assay and represent the DNA relatedness (%) found between different *Bdellovibrio* strains. Complete (100±0%) hybridization corresponds to the intensity measured when labelled genomic DNA is hybridized to itself. (Baer *et al*., 2000)

Labelled strain	*B. bacteriovorus*					*B. stolpii* Uki2^T	*B. starrii* A3.12^T
	100^T	109J	Ox9-2	E	2484Se2		
B. bacteriovorus 100^T	100·0	100·0	72·2	96·0	44·8	2·6	4·4
B. bacteriovorus 109J	100·0	100·0	88·2	91·0	32·1	3·1	2·0
B. bacteriovorus Ox9-2	75·0	87·5	100·0	92·1	52·6	2·1	3·7
B. bacteriovorus E	94·5	89·4	88·5	100·0	32·6	2·9	1·9
B. bacteriovorus 2484Se2	41·7	35·2	54·7	36·4	100·0	0·0	2·2
B. stolpii Uki2^T	2·8	2·2	2·5	2·3	0·0	100·0	4·2
B. starrii A3.12^T	4·1	2·5	3·1	3·0	3·1	4·0	100·0

In a similar experiment, phylogenetic analysis of 17 freshwater and 9 marine BALOs was carried out by Synder *et al*. (2002) and their results showed that the aquatic BALOs formed two clusters. The first clusters include *B. bacteriovorus* which composed mostly of the freshwater and terrestrial isolates while the second clade of BALOs include the freshwater *Bacteriovorax starrii*, *Bacteriovorax stolpii* and nine marine isolates referred to as saltwater isolates. Based on the work of Seidler *et al*. (1972), Baer *et al*. (2000), and Synder *et al*. (2002) the total number of Genus in the group δ-BALOs now became two viz *Bdellovibrio* and *Bacteriovorax.* However, the research on these three groups were limited to BALO isolates isolated from only one particular habitat (soil and aquatic habitats). Davidov and Jurkevitch (2004) showed the great phylogenetic diversity within the group *Bdellovibrio* and *Bacteriovorax* after carrying out amplified rDNA restriction analysis of different BALO strains isolated from different habitats and geographical areas. They further proposed the establishment of new genus *Peredibacter* for *Bacteriovorax starrii* (now *Peredibacter starrii*) as well as the regrouping of *Bdellovibrio* and *Bacteriovorax-peredibacter* lineage into two different families, i.e. *'Bdellovibrionaceae'* and a new family, *Bacteriovoracaceae*. In addition to phylogenetic and ribotyping analysis, Schuwdke *et al*. (2001) characterized different BALO strains including ones isolated from guts of man and other animals by carrying out the amplification and hybridization experiment with host interaction (*hit*) probe. Host interaction (*hit*) locus has been

described to play important role during *B. bacteriovorus* attack on prey (Cotter and Tomashow, 1992; Schuwdke *et al*., 2001). From the results of Schwudke and his colleagues, all the *Bdellovibrio* strains isolated from guts of animal showed positive signal in the hybridization experiment with the *hit* probe. On the contrary, the strains of *Bacteriovorax* and *Bdellovibrio* sp. W, a strain that has been reported to produce Bdellocyst (Tudor and Conti, 1978) were observed to be negative for the *hit* probe hybridization experiment. They then concluded that the *hit* locus is restricted to the *B. bacteriovorus* strains and can be used as probe for the detection of this species.

The freshwater/terrestrial and marine BALOs used to be grouped together under the same genus *Bdellovibrio* (Baer *et al*., 2004). However, marine BALOs have been reported to differ based on prey range, salinity and temperature growth ranges (Marbach *et al*., 1975; Sutton and Besant, 1994). The marine or saltwater BALOs were later reclassified to the genus *Bacteriovorax* because of the relatedness they shared together based on the 16S rDNA and two species including *Bacteriovorax marinus* sp. nov and *Bacteriovorax litoralis* sp. nov were proposed (Baer *et al*., 2004). Recently, the two saltwater *Bacteriovorax* (*B. marinus* and *B. litoralis*) were placed into a new genus *Halobacteriovorax* (Ha.lo.bac.te.ri.o.vo'rax.: Gr. n. hals, halos salt; L. neut. n. bacterium, a small rod; L. adj. vorax, devouring, ravenous, voracious; N.L. masc. n. *Halobacteriovorax*, devourer of bacteria in saltwater environments). The reasons for the reclassification is because they are the only family with members found in the salt-water environment (Koval *et al*., 2015). Summarily, based on phylogenetic tree construction, the *Bdellovibrio* and *Halobacteriovorax* were taken to be the distant taxa while the *Bacteriovorax* and *Peredibacter* are the intermediates.

2.1.4. ECOLOGY AND QUANTIFICATION OF BALOS

Bdellovibrio spp. are ubiquitous and widely distributed in nature. *B. bacteriovorus* was first isolated from soil (Stolp and Petzold, 1962). Jurkevitch *et al*., (2000) characterized and observed that *Bdellovibrio* spp isolated from soil, rhizosphere, and roots of beans and tomatoes exhibited genetic variation based on 16S rRNA gene and *hit* locus. Uematsu (1980) isolated *Bdellovibrio* spp. from rice paddies in Japan. Also, *Bdellovibrio* spp. have been isolated from different water systems including artificial water system (Richardson, 1990), seawater (Sutton and Besant, 1994), estuaries

(Williams *et al*., 1980), gills of blue crab (Kelley and Williams, 1992) and oyster shells (Kelley *et al*., 1997). Ibragimov (1980) reported that *B. bacteriovorus* are present in the intestine of cows, horses, pigs and ducks, and constantly released with the faeces of the animals into the environment. However, he was unable to detect *B. bacteriovorus* in the faeces of man, white mice, frogs and fishes. On the contrary, higher abundance of *B. bacteriovorus* in the guts of healthy humans was reported by Lebba *et al*., (2013).

Enumeration of *Bdellovibrio* spp. by plaque formation and microscopy have several draw backs. These draw backs include the long period of time needed for the appearance of *Bdellovibrio* plaques and the inability of culture dependent technique to detect all *Bdellovibrio* strains in the environment. Furthermore, some *Bdellovibrio* strains might require unculturable prey bacteria to survive. The minute size and the high motility of *Bdellovibrio* spp is a limitation to the enumeration of *Bdellovibrio* spp. using microscopy techniques. Therefore, different culture-independent methods that can be used for efficient quantification of member of the Bdellovibrionaceae have been described. One of the methods is the use of Fluorescence In Situ Hybridization (FISH) described by Mahmoud *et al*., 2007. This technique relies on the use of epifluorescence microscopy or confocal scanning laser microscopy to observe fluorescencely labeled oligonucleotide probes specific for rRNA sequences of certain groups of bacteria. Mahmoud *et al*., (2007) was able to obtain an intense fluorescence signal for cells growing in the Bdelloplast using FISH. They further attributed the low intensity signals obtained from released attack-phase cells to probably be as a reult of the depletion of cellular rRNA content. They finally suggested the use of the FISH technique to study the abundance and ecological roles of *Bdellovibrio* spp. in the environment. Furthermore, the detection and characterization of BALOs in the environment is now posible with the availability of taxón-specific 16S rDNA taxon-specific PCR primers (Jurkevitch and Ramati 2000). The use of quantitative PCR (qPCR) to enumerate salt-water *Bacteriovorax* has been described (Zheng *et al*., 2008). Similarly, Van Essche *et al*., (2009) developed a Bdellovibrionaceae-specific primers and a Bdellovibrionaceae-specific probe for the enumeration of the members of the Bdellovibrionaceae in fresh wáter samples using qPCR technique.

2.1.5. *BDELLOVIBRIO BACTERIOVORUS*

Bdellovibrio bacteriovorus was accidentally discovered in 1962 by Stolp and Petzold form soil while attempting to isolate bacteriophages for the biocontrol of phytopathogens. The word "*Bdello"* is a Greek word which means "leech" and "vibrio describes the "comma" or curve shape of *Bdellovibrio*. The word "bacteriovorus" was coined because of its ability to "eat" other bacteria. *B. bacteriovorus* is a tiny Gram-negative bacterium that possesses the fascinating ability to prey upon other Gram-negative bacteria. It utilizes the cellular material of its prey for growth and production of progenies. *B. bacteriovorus* belongs to the phylum proteobacteria, Class deltaproteobacteria, and Order Bdellovibrionales. It is further classified into the Family Bdellovibrionaceae, Genus *Bdellovibrio* and Species *bacteriovorus*.

B. bacteriovorus has a size ranging from 0.2-0.5 µm × 0.5-2.5 µm. It is highly motile (160 µm s^{-1} for *B. bacteriovorus* HD100) (Sockett and Lambert, 2004) with a long, thick, single polar flagellum of about 50 mµ in diameter (Stolp and Starr, 1963). The flagellum is sheathed with a membranous material continuous with the outer membrane. The flagellum is irregularly shaped with a tapered wavy filament; it has three or four waves with smaller waves closed to the distal part (Lida *et al*., 2009). In *B. bacteriovorus* 109J and HD100, there are six different flagellar filament protein (flagellins *fli*C1 to *fli*C6) present in their genomes (Lambert *et al*., 2006).

The cell wall of *B. bacteriovorus* is made up of thin layer of peptidoglycan as observed in all Gram-negative bacteria. However, instead of lipopolysaccharides that are usually present in Gram-negative bacteria, the cell envelope of *B. bacteriovorus* is made up of large amount of sphingolipids in the form of sphingophosphonolipids (Watanabe et al., 2001; Ikushiro et al., 2007).

2.1.6. LIFE CYCLE OF *BDELLOVIBRIO BACTERIOVORUS*.

The life cycle of *B. bacteriovorus* is complex involving two distinct phases; the attack phase which involves small, obligate, flagellated, vibroid *B. bacteriovorus* rapidly and randomly swimming

to seek a suitable prey. Upon successful location of the prey, the second phase is initiated with *B. bacteriovorus* penetrating into the periplasmic space of the prey (Figure 2. 6). During the latter phase, *B. bacteriovorus* digest prey cellular materials, utilizes it, and divides by septation to release new progenies for the attack phase. In an *in vitro* laboratory set-up, a life cycle of *B. bacteriovorus* is expected to be completed within 3-4 h (Tudor and McCann, 2006).

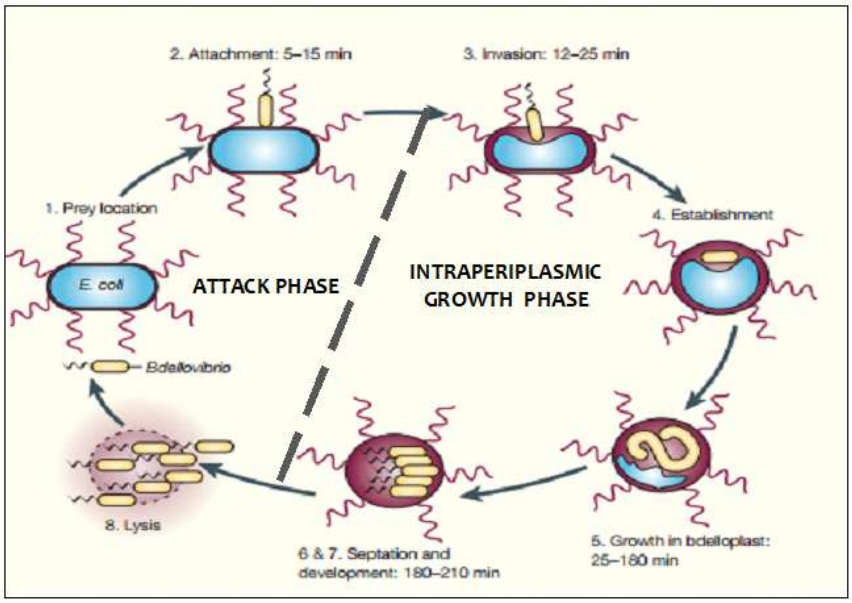


Figure 2. 6. The predatory life cycle of *B. bacteriovorus* showing the two stages - attack and intraperiplasmic phases and the time required to complete each stage. (Sockett & Lambert, 2004)

2.6.1. Events during the attack phase of *B. bacteriovorus* life cycle

The survival of the attack phase of *B. bacteriovorus* depends on its ability to seek and locate an appropriate prey. *B. bacteriovorus* swim rapidly in liquid medium to find a prey with the aid of its single sheathed polar flagellum. The process of prey location is a highly energetic process with *B. bacteriovorus* possibly losing its viability in the absence of prey. The rate of endogenous respiration of *B. bacteriovorus* during this process has been described to be about seven times that of *Escherichia coli* (Hespell *et al*., 1973). The factors that attract *B. bacteriovorus* to prey-rich region has not been successfully determined. However, reports suggested that it chemotactically responds towards oxygen and compounds such as amino acids. Furthermore, predation has been reported to be reduced when there is distruption in the genes encoding Methyl-accepting

chemotactic proteins (MCP). Prior to invasion, *B. bacteriovorus* collides and attaches reversibly to the prey's external surface via the pole opposite the flagellum, using thin, flexible fibre structure called type IV pilus. The mechanism of predator-prey interaction has been suggested to involve lipopolysaccharide interaction (Schelling and Conti, 1986). In addition to the attachment, the flagellum of B*. bacteriovorus* has been reported to be completely lost or shed before invasion starts (Thomashow and Rittenberg, 1972). On the contrary, Lambert *et al*. (2006) in their experiment involving the predation of *B. bacteriovorus* 109J on *Escherichia coli fliG* null strain DFB225 (a strain that is not capable of synthesizing its own flagellar filament) showed that flagellum is not always shed when *B. bacteriovorus* penetrates its prey.

After a reversible attachment and a short period of recognition, *B. bacteriovorus* irreversibly attaches to the prey and finally uses the twitching-pilus to penetrate into the prey's periplasmic space to commence the intraperiplasmic developmental stage. The type IV pilus system has been reported to be crucial for the attachment and invasion of *B. bacteriovorus* (Evans *et al*., 2007). Predation in *B. bacteriovorus* 109J was observed to be inhibited and delayed when antibodies were used against the type IV pilus (Mahmoud and Koval., 2010).

During the penetration, *B. bacteriovorus* generates a small pore on the prey's cell wall, providing an opportunity for *B. bacteriovorus* to squeeze itself into the already expanded periplasmic space. The mechanism through which the pore is formed has been suggested to either involve the enzymatic digestion (Huang and Starr, 1973) or mechanical drilling (Starr & Baigent, 1966) of the prey cell wall. In *B. bacteriovorus* HD100 strain, the formation of the penetration pore has been attributed to the activity of a localized peptidase anchored in the outer membrane of the anterior pole of the predator. The peptidase encoded by gene *Bd0168* is known to produce a localized hydrolysis of the prey peptide cross links thereby leading to the pore formation. However, during the pore formation and invasion, it is important for the prey cell wall to maintain a certain degree of integrity so that *B. bacteriovorus* will be able to successfully establish itself in the periplasmic space. *B. bacteriovorus* has been reported to produce glycanases and peptidases that help to prevent excessive prey cell damage and loss of cellular constituents (Thomashow and

Rittenberg, 1978a, b, c; Mahmoud and Koval., 2010). Once *B. bacteriovorus* has been able to establish itself in the periplasm, the pore formed is then re-sealed.

2.1.7. Events during the intraperiplasmic invasion phase of *B. bacteriovorus* life cycle

Upon successful *B. bacteriovorus* penetration, the prey becomes immobilized, and its important metabolic activities such as cellular respiration, RNA and protein synthesis are arrested (Rittenberg and Shilo 1970; Thomashow and Rittenberg, 1978a). *B. bacteriovorus* then attaches to the prey cytoplasmic membrane, and the prey's cell loses its rigidity and undergoes morphological changes to form a round osmotically stable structure known as Bdelloplast.

The formation of Bdelloplast in *B. bacteriovorus* has been linked with glycanase activity and the hydrolysis of the cross-linking peptides in the prey cell (Tudor *et al*., 1990). However, Lambert *et al*. (2015) described two *B. bacteriovorus* peptidoglycan DD-endopeptidases encoded by gene *Bd0816* and *Bd3459* as enzymes involved in bdelloplast formation. The enzymes modify the prey cell wall by hydrolysis of the structural 3-4 peptide crosslinks (Lerner *et al*., 2012) ultimately preventing subsequent invasion of the already invaded prey by another *B. bacteriovorus*. Another important event that occurs during the invasion process is how *B. bacteriovorus* protects itself from the peptidoglycan DD-endopeptidases. *B. bacteriovorus* has been reported to protect itself from the bdelloplast-forming endopeptidases via a small ankyrin repeat protein encoded by gene *Bd3460* in *B. bacteriovorus* HD100 strain (Lambert *et al*., 2015). The Bd3460 protein binds the Bd0816 and Bd3459 endopeptidases via a common epitope to form complexes that prevents cell wall decrosslinking in *B. bacteriovorus*. Lambert *et al*. (2015) further demonstrated that *B. bacteriovorus* that lack this protection can round up into a non-invasive spheroplast-structure and die when the endopeptidases are released during predator-prey contact (Figure 2. 7).

In the osmotically stabled Bdelloplast, the prey cell is usually killed within 10-20 min after penetration. *B. bacteriovorus* then uses different hydrolytic enzymes to degrade the cellular macromolecules including DNA, RNA, sugars and proteins in a regulated manner. During the intraperiplasmic phase, the protease activity has been reported to increase throughout the stage (Romo *et al*., 1992). The solutes generated are absorbed by *B. bacteriovorus* through Adenosine

triphosphate (ATP) – binding cassette (ABC) and Major Facilitator Superfamily (MFS) transport system (Rendulic *et al.*, 2004). Rendulic *et al*. (2004) further suggested that *B. bacteriovorus* build up their cellular components from the end products of prey digestion using the prey's metabolic machineries. When the prey cellular components are exhausted, *B. bacteriovorus* elongates at the non-flagellated end into a spiral-shaped structure that undergoes septation and fragmentation to produce flagellated progenies. Finally, lytic enzymes are released to digest the remaining bdelloplasts thereby leading to the release of the flagellated progenies that will launch new attack-phase.

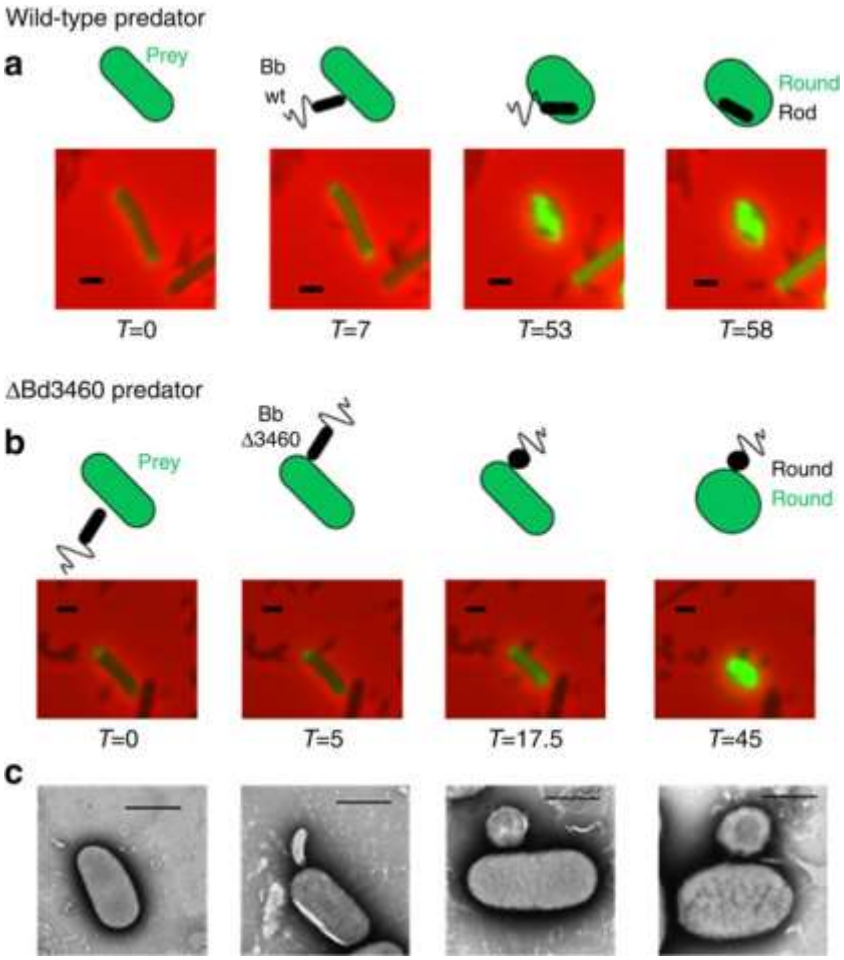


Figure 2. 7. ΔBd3460 *Bdellovibrio* self-round upon initiating prey cell entry.
Epifluorescence phase contrast microscopy of *Bdellovibrio* (small, phase dark, comma-shaped cells) preying upon *E. coli* prey cells which have periplasms constitutively fluorescently labelled by a pMal::mCherry fusion. A cartoon representation is presented above each. (**a**) Control using host independent strain HID22 which is wild-type for Bd3460 (Bb wt) and shows typical attachment to and entry into the prey cell which is rounded up in the process. (**b**) ΔBd3460 host independent strain (Bb Δ3460) attaches to the prey cell in a manner similar to the wild-type control, but then rounds up itself, preventing entry into the prey cell. (**c**) Representative electron micrographs showing the different stages of attachment, *Bdellovibrio* rounding, and prey rounding. Scale bars, 1 μm; time is indicated in minutes. (Lambert *et al*., 2015)

2.1.8. APPLICATIONS OF *BDELLOVIBRIO BACTERIOVORUS*

B. bacteriovorus degrade bacterial prey cellular materials while residing in their (prey) intracellular space leading to prey death and reduction in population. This unique characteristic of *B. bacteriovorus* makes it a potential biocontrol agent against pathogenic bacteria in different fields including medicine, agriculture and food industries.

The first application of *B. bacteriovorus* as biocontrol agent was done by Scherff in 1973. He demonstrated the ability of *B. bacteriovorus* Bd-17 to effectively inhibit the development of local and systemic symptoms of bacterial blight of soybean caused by *Pseudomonas glycinae*. However, in the same experiment, two other *B. bacteriovorus* strain Bd-10 and Bd-19 were less effective in the control of the bacterial blight disease in soybean. The experiment further reported a close correlation between the cell burst size (number of progenies released after infection) and complete inhibition of bacterial blight. The experiment of Scherff was based on co-inoculation of the *B. bacteriovorus* with the *P. glycinae* on soybean plant. It will however be important to study the effect of *B. bacteriovorus* in a post-infection treatment (Sockett & Lambert, 2004). In addition, several reports have also shown the potentials of *B. bacteriovorus* in the control of phytopathogens of agricultural importance such as *Pectobacterium atrosepticum* that causes soft-rot disease in

potatoes (Epton *et al*., 1989), *Erwinia carotovora*, *Burkholderia* spp., *Xanthomonas* spp., *Pseudomonas* spp and *Agrobacterium* spp. (Uematsu, 1980; Jurkevitch *et al*., 2000; Song, 2004; McNeely *et al*., 2016).

In animal husbandry, *B. bacteriovorus* has shown potent activity against pathogenic bacteria. *B. bacteriovorus* 109J was shown to effectively control *Moraxella bovis* infection of bovine cornea in an in vitro model of infectious bovine keratoconjunctivitis. (Boileau *et al*., 2011). The *Bdellovibrio* strain suspension (1.6×10^{11} PFU/mL) was reported to decrease adherence of *M. bovis* (4×10^{7} CFU/mL) to Madin-Darby bovine kidney cells by 6-fold at 12 h treatment, and reduced the number of unattached *M. bovis* cells by 1.4 folds. Unfortunately in an in vivo study, *B. bacteriovorus* 109J was not effective in the treatment of infectious bovine keratoconjunctivitis and at the sametime observed not to persist in the tears of calves with experimentally induced infectious bovine keratoconjunctivitis (Boileau *et al*., 2016). However, the same research group earlier in an unpublished experiment report stated that *B. bacteriovorus* is not pathogenic to bovine eyes in vitro and can persist on the corneal surface of cattle for a mean of 6 days. In another study, *B. bacteriovorus* HD100 was observed to reduce the burden of *Salmonella* sp. in cattle faeces and rumen fluids, and therefore suggested its use as a preharvest intervention to control pathogenic *Salmonella* sp. that can contaminate cattle hides and carcasses (Page *et al*., 2015). Furthermore, the inability of *B. bacteriovorus* HD100 to reduce bacterial population of *E. coli* O157:H7, as observed in the same study, was hypothesized to be as a result of *E. coli*'s ability to produce indole which can reduce *B. bacteriovorus*'s predation as earlier reported (Dwidar *et al*., 2015).

In an effort to study the possible application of *B. bacteriovorus* in control of foodborne pathogen and food spoilage bacteria, Fratamico and Cooke (1996) isolated and studied *B. bacteriovorus* strain 45k that was capable of lysing *E. coli* 0157:H7 strain 45753-35 and serotype 026:H11 strain 2239-69 dried onto stainless steel usually used in food industries for packaging and processing of food materials. The ability of the *B. bacteriovorus* strain 45k to lyse biofilms formed by *E. coli* 45753-35 was also reported. In an earlier study by Fratamico and Whiting (1995), they observed the inability of *B. bacteriovorus* 109J to decrease the population of *E. coli* serotype

0157:H7. In comparison, the variation in the predation pattern of *B. bacteriovorus* HD100, 45k and 109J on *E. coli* serotype 0157:H7 as observed in the studies done by Page *et al.* (2015), Fratamico and Cooke (1996), and Fratamico and Whiting (1995) revealed the non-specific prey range attribute of *B. bacteriovorus*. In an experiment performed by Varon and Shilo (1969), *Salmonella* spp. and *E. coli* mutants lacking the O – specific side chains but containing a complete "rough" core (R core) were observed to be better receptors than their wild-type (smooth) strains. Varon and Shilo (1969) further reported the R antigen portion of the prey lipopolysaccharide as a possible location for the receptor for *B. bacteriovorus*. However, there is still need for more studies to further understand the specific predator receptor on the prey of *B. bacteriovorus*.

Food spoilage is a major problem because of its environmental and economic implications. The potentials of predatory bacteria such as *B. bacteriovorus* and *Micavibrio* spp. as biocontrol agents for foodborne and plant pathogens including food spoilage bacteria was substantially reviewed by Olanya and Lakshman (2015). *Pseudomonas tolasii*, an important bacterial pathogen that is implicated in spoilage of mushroom was successfully controlled by adding co-inoculating it with *B. bacteriovorus* HD100 on post-harvest mushroom (Saxon *et al*., 2014).

B. bacteriovorus has been reported by several studies as an alternative to antibiotics in the treatment or protection of fishes and shrimps against bacterial pathogens. Chu and Zhu, (2010) suggested the possibility of using *B. bacteriovorus* to control infection caused by *Aeromonas hydrophilia* in fishes. Similarly, a *B. bacteriovorus* strain F16 isolated from a sturgeon gut was able to lyse thirteen pathogenic strain of *Aeromonas* species that are capable of causing infections in fishes (Cao *et al*., 2012). The research also showed that *B. bacteriovorus* F16 is safe as a biocontrol agent in aquaculture because the test mice and sturgeons in the study exhibited no visible signs of disease or mortality. Furthermore, the positive protective effect of *B. bacteriovorus* strain H16 in controlling infections caused by *Proteus penneri* (Cao et al., 2014) and *Vibrio cholera* (Cao *et al*., 2015) in white shrimps (*Penaeus vannamei*) have been reported. The potential of BALOs as probiotics in aquaculture has also been suggested (Guo *et al*., 2016; Guo *et al*., 2017).

The first attempt to study the potentials of *B. bacteriovorus* for waste water treatment was reported by Venosa (1975). In the study, *B. bacteriovorus* strains 109D, 110 and 114 were able to lyse strains of smooth or S type free-living *Sphaerotilus natans* that are problematic bacteria during waste water treatment. The limitation of the application observed in the work was that the *B. bacteriovorus* strains were only able to attach to the filamentous form of *S. natans* but unable to penetrate its protective sheath. *B. bacteriovorus* has also been reported to produce heat-stable antiagal factor which acts to inhibit the photosynthesis in *Phormidium luridum*, blue-green algae that can pose a problem in freshwater habitat (Burnham *et al.*, 1976). This research showed that the application of *B. bacteriovorus* could not only be limited to the biocontrol of pathogenic Gram negative bacteria. Membrane biofouling is one the major challenges encountered during waste water treatment with membrane bioreactors. Membrane biofouling is caused by microorganisms that excrete complex compounds or develop biofilms that can plug waste water plant filter. The flux of filters in waste water treatment plant has been reported to be improved by applying *B. bacteriovorus* to lyse bacteria or biofilms that can plug membrane filters (Kim *et al*., 2013; Yilmaz *et al*., 2014). Recently, the ability of *B. bacteriovorus* to induce biolysis of sludge in order to promote dewaterability during waste water treatment was reported (Yu *et al*., 2017).

Researchers have demonstrated wide applications of *B. bacteriovorus* in the medical field. Some pathogenic strains of bacteria such as members of the genus *Acinetobacter*, *Aeromonas*, *Bordetella*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Listonella*, *Morganella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella* and *Vibrio* have been shown to be susceptible to predation by *B. bacteriovorus* (Dashiff *et al*., 2011). The emergence of multidrug-resistant bacteria and reduction in the rate at which new antibiotics agents are discovered are a major global concern most especially in the healthcare settings (Tan *et al*., 2013; Safdar *et al*., 2017). The ability of bacteria to develop different mechanisms of resistance to antibiotics including recently developed antimicrobials calls for the development of alternative therapy or treatment for infections caused by multidrug-resistant bacteria. *B. bacteriovorus* 109J and HD100 have been shown to prey upon multidrug-resistant pathogens of humans including *Acinetobacter baumanii*, *E. coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* (Kadouri *et al*., 2013; Sun *et al*., 2017).

Most scientific publications have limited the prey range of *B. bacteriovorus* to Gram negative bacteria (Hobley *et al*., 2006; Sockett, 2009; Essche *et al*., 2009; Atterbury *et al*., 2011). However, by using field emission scanning electron microscope, Lebba *et al* (2014) have been able to demonstrate that *B. bacteriovorus* HD100 is capable of using epibiotic mode of predation to attack *Staphylococcus aureus*, a Gram positive bacterium (Figure 2. 8).

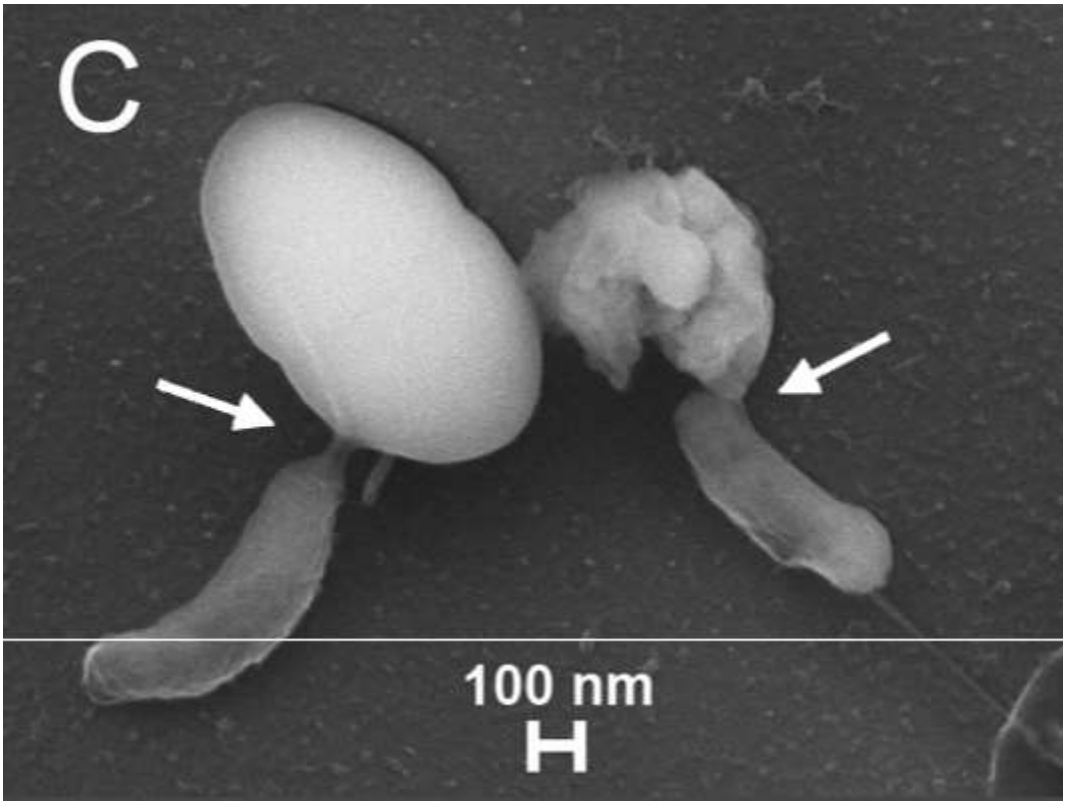


Figure 2. 8. Predation of *B. bacteriovorus* on 'static' biofilm of *S. aureus.* SEM images of *S. aureus* biofilm (panel A, 30000X) grown for 24 h on a silicon plate, and after 24 h of *B. bacteriovorus* HD100 predation (panel B, 20000X). A free *Bdellovibrio* is visible with its long polar flagellum (estimated length, 4 µm) (arrow, panel B, inset, 50000X). A higher SEM magnification (88830X) shows the initial attack phase of HD100 on a prey cell (panel C, left) and the late attack phase, with a destroyed *S. aureus* cell (panel C, right). White arrows in panel C show the direct interaction of *B. bacteriovorus* HD100 with *S. aureus*. (Lebba *et al*., 2014).

The potentials of *B. bacteriovorus* to treat eye infections was first reported by Nakamura (1972). In the study, Nakamura showed that *B. bacteriovorus* is capable of reducing the severity of

keratoconjunctivitis induced by *S. flexneri* in rabbit eyes. He further showed that treatment of keratoconjunctivitis is better when *B. bacteriovorus* is administered within 48 h of *Shigella* infection compared to when administered after 72 h of infection. In an in vitro experiment, *B. bacteriovorus* HD100 and 109J were able to lyse *Serratia marcescens* and *Pseudomonas aeruginosa*, common ocular bacterial pathogens isolated from keratitis patients.

The effects of *B. bacteriovorus* on the complex polymicrobial oral pathogens have been studied and report showed that *B. bacteriovorus* is capable of causing significant reduction in the population of pathogens such as *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens* and *Fusobacterium nucleatum* (Essche *et al*., 2009; Essche *et al*., 2011; Loozen *et al*., 2015). The ability of *B. bacteriovorus* to further lyse the biofilms of oral pathogen, survive in saliva and oxygen-limited conditions of the oral cavity (Dashiff & Kadouri, 2011) further indicates the possibility of its successful application as agent of biocontrol against oral pathogens.

The ultimate goal of finding applications of *B. bacteriovorus* is gradually advancing with several studies in animal models yielding positive and promising results. *B. bacteriovorus* HD100 was observed to significantly reduce the burden of pathogenic *Salmonella enteritidis* and inflammation in the guts of chicks when orally dosed (Atterbury *et al*., 2011). In addition, several other studies have also shown that *B. bacteriovorus* are potent against multidrug-resistant and non-resistant bacteria models (Dashiff *et al*., 2011; Kadouri *et al*., 2013; Sun *et al*., 2017) with a significant level of safety in the animal. The administration of *B. bacteriovorus* HD100, 109J and *Micavibrio aeruginosavorus* to monolayers of human keratocytes did not produce any damaging effects (Romanowski *et al*., 2016). The safety of the predatory bacteria (*B. bacteriovorus* HD100, 109J and *Micavibrio aeruginosavorus*) was further tested in the same experiment by applying them to the cornea of New Zealand white rabbits. In comparison with vancomycin, the predatory bacteria were non-toxic. However, the vancomycin was toxic, inducing inflammation and swelling of the conjunctiva and production of ocular mucous. The ability of the predatory bacteria to cause reduced inflammatory response were suggested to be as a result of their unique structure of the lipid A portion of their lipopolysaccharide (Schuwdke *et al*., 2003) and the possession of sheathed flagella

(Thomashow & Rittenberg, 1978) which could not interact with Toll-receptor 4 and 5 (TLR4 and TLR5) respectively. Romanowski and colleagues further showed that aside the fact that the predatory bacteria were tolerated in vivo, they did not produce any effects that can impede corneal epithelial wound healing at the ocular site of the rabbits, unlike vancomycin which is usually applied to control ocular infections.

In another experiment that was recently conducted by Shatzkes *et al*. (2016), the non-toxic characteristics and the ability of *B. bacteriovorus* and *M. aeruginosavorus* to reduce bacterial burden in mammalian system in vivo were further supported. The predatory bacteria were observed to reduce the burden of bacteria in the lungs of rats by a mean of 2 logs when the rats were challenged with a sublethal doses of *K. pneumoniae* via intranasal inoculation. There was initial evidence of rise in inflammatory response at 24 h post-infection when the predatory bacteria were administered, however, the response was not strong enough to cause damage to the rat's tissues. Furthermore, the ability of *B. bacteriovorus* and *M. aeruginosavorus* to reduce microbial burden when administered intravenously into rats was reported by Shatzke *et al*. (2017). The group observed no rat morbidity or damage to the various organs. There was an increase in proinflammatory cytokines (TNFα and KC/GRO) at 2 h post-inoculation; however, cytokines returned to baseline levels by 18 h. The study concluded that the predatory bacteria may not be effective for the treatment of acute blood infections because they are unable to significantly reduce the burden of *K. pneumoniae* in the blood or prevent its dissemination to other organs. In a recent study, serum albumin and osmolality have been reported to inhibit *B. bacteriovorus* predation in human serum (Im *et al*., 2017). However, intrarectal inoculation of *B. bacteriovorus* into the gastrointestinal of rats have been reported to be associated with healthy benefits while *M. aeruginosavorus* exhibited potential adverse effects on gut microbiota (Shatzkes *et al*., 2017). Furthermore, the ability of *B. bacteriovorus* to work synergistically with host immune response in the killing of antibiotic-resistant human pathogen *Shigella flexneri* was reported in an in vivo study carried out using zebra fish (*Danio rerio*) larvae (Willis *et al*., 2016). In accordance with several other studies, this study also showed that *B. bacteriovorus* is safe to be used as an antibacterial agent.

In human cell line, *B. bacteriovorus* and some other predatory bacteria have been shown to be non-cytotoxic or non-pathogenic suggesting their potential use as live antibiotics against human pathogens (Gupta *et al*., 2016; Monnappa *et al*., 2016).

The application of *B. bacteriovorus* is not only limited to its use as an antibacterial agent. It can be used as a novel, biological lytic agent for the inexpensive, industrial-scale recovery of intracellular products such as polyhydroxyalkanoates from different Gram-negative prey cultures (Martinez *et al*., 2016) (Figure 2. 9).

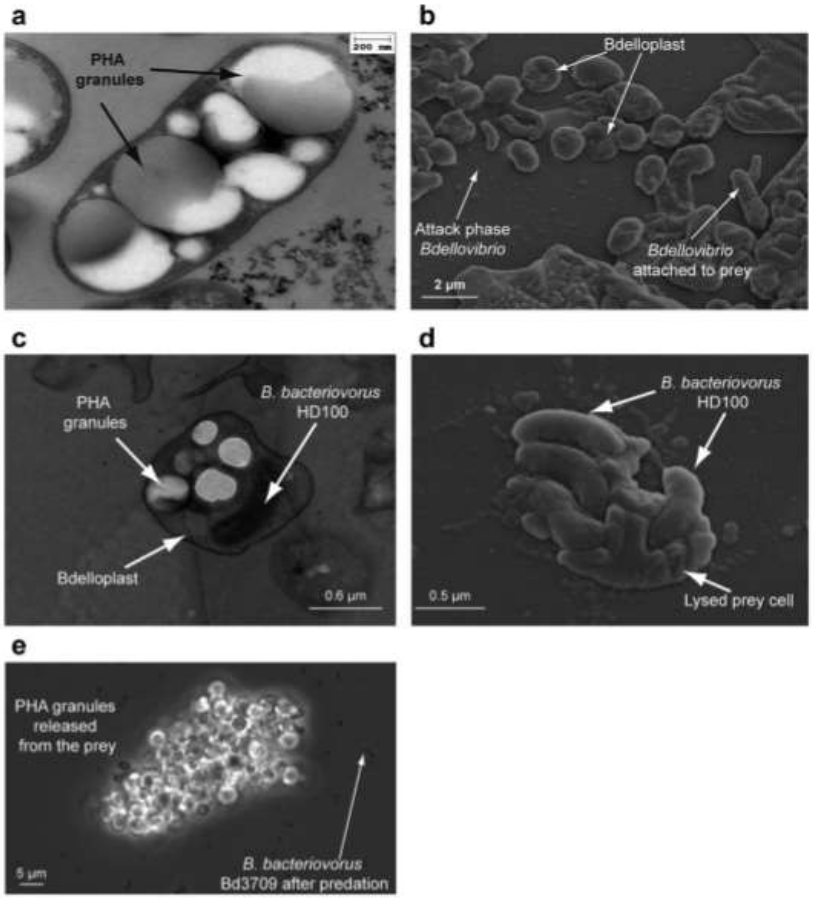


Figure 2. 9. Different growth stages of B. bacteriovorus HD100 preying on *P. putida* KT2440. (a) TEM image of *P. putida* KT2440 accumulating mcl-PHA. (b) SEM image of a co-culture of *B. bacteriovorus* HD100 preying on *P. putida* KT2440. Different predator growth stages can be distinguished: Attack phase predator cells, entering the periplasm of the prey and growing in rounded prey cells (bdelloplast). (c) Detailed TEM image of predator cell development within a bdelloplast. (d) Detailed SEM image of prey cell lysis and release of predator progeny into the

medium. (e) PHA granules released by *B. bacteriovorus* Bd3709 mutant after 24 h of predation upon *P. putida* KT2440 (Martinez *et al.,* 2016).

2.1.9. PROS AND CONS OF *BDELLOVIBRIO BACTERIOVORUS* APPLICATION

The beguiling life style of *B. bacteriovorus* that makes it an important potential bacteria for control of pathogenic and antibiotic-resistant bacteria is accompanied with different limitations that can make its applications a herculean task for scientists. Firstly, *B. bacteriovorus* does not completely kill the prey even at high predator to prey ratios. Prey resistance to *B. bacteriovorus* has been described as plastic (not genetically encoded) (Shemesh & Jurkevitch, 2004). However, *B. bacteriovorus* has been reported to be intrinsically resistant to the beta-lactam antibiotics (Thomashow & Rittenberg, 1978; Sockett & Lambert, 2004) suggesting the possible synergistic effects of *B. bacteriovorus* and antibiotics in eradication of pathogens. In any case, the plastic resistance suggests that prey will always be susceptible to *B. bacteriovorus*. Furthermore, *B. bacteriovorus* has broad host range which raises the question of the fate of beneficial bacteria in an environment such as guts that has mixed bacterial cultures. The presence of other bacteria might even affect the rate of predation positively or negatively (Harini *et al*., 2013).

The predatory activities of *B. bacteriovorus* in an anaerobic environment such as gut or urinary tract might be a concern because *B. bacteriovorus* has been described as a strict aerobe (Simpson & Robinson, 1968; Fry & Staples, 1976). However, the ability of *B. bacteriovorus* to survive in microaerophilic condition and in anoxic for nine days has been reported (Schoeffield *et al*., 1996). *B. bacteriovorus* also has challenge of inability to prey upon Gram negative bacteria with S-layer on their surfaces (Koval & Hynes, 1991). Also, *B. bacteriovorus* activity is decreased in the presence of some chemicals, such as high concentrations of glucose or glycerol, and at low pH (Dashiff *et al*., 2011).

Finally, *B. bacteriovorus* remains promising as a living antimicrobial because it has been shown to have predatory capabilities over pathogens without adverse effects on humans.

CHAPTER THREE

3.1. GENERAL DESCRIPTION OF TECHNIQUE FOR ISOLATION OF *BDELLOVIBRIO* SPP FROM SOIL AND SEWAGE.

Bdellovibrio spp. have been isolated from quite a number of habitats including freshwater, marine, soil, plant rhizosphere, and guts of animals (Jurkevitch *et al*., 2000; Williams *et al*., 1995; Schwudke *et al*., 2001). They have also been reported to be abundant in the guts of man (Lebba *et al*., 2013). Several studies have been done on *Bdellovibrio* spp. However, as at the time of this study and to the best of our knowledge, this is the first time that studies on *Bdellovibrio* spp. isolated from soil and sewage is reported in Mexico. The technique for the isolation of *Bdellovibrio* spp. is relatively complex. The reason is that *Bdellovibrio* spp. are obligate predatory bacteria (except for *B. bacteriovorus* strain Tiberius that was observed to grow simultaneously as predatory and prey-independent strain in nature) and cannot grow on nutrient rich media except in the presence of a suitable prey. Secondly, successful isolation of *Bdellovibrio* spp. from environmental samples requires that they are separated from other environmental microorganisms most especially predatory ones such as protists, and bacteriophages. The process of separating *Bdellovibrio* spp. from other environmental bacteria depends on the sample. For sewage sample, centrifugation is done first at relatively low revolution per minute (rpm) between 3000 – 5000 rpm to separate larger particles and bacteria. The supernatant is then centrifuged at high revolution per minutes (27000 xg ~ 15000 – 17, 000 rpm) to reduce the number of bacteriophages. The next step is to resuspend the pellet and filter through 0.45 µm syringe filter (sometimes serial filtration through filter of different pores sizes; 3.0 µm, 1.2 µm, 0.8 µm, 0.65 µm and 0.45 µm can be done to minimize filter clogging) to eliminate other remaining bacteria. Soil samples are usually suspended in sterile water or suitable buffer (HEPES buffer; (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) and shaken for 1 – 2 h before carrying out centrifugation at low rpm. The use of large sample sizes can cover for the loss of *Bdellovibrio* spp. during the centrifugation and filtration processes. Alternatively, the population of *Bdellovibrio* spp. can be increased by enriching samples with prey cells.

Sometimes, *Bdellovibrio* spp. can be present in low numbers in a particular environment. Enrichment method is used to increase the cell numbers of the *Bdellovibrio* spp. before isolation. Also, this method can be employed when *Bdellovibrio* spp. that can prey on specific species of bacterium is of interest. Enrichment method involves culturing a prey bacterium in a nutrient rich broth and washed in a suitable buffer or medium. The washing process include centrifugation of grown prey broth culture, and the pellet obtained is resuspended in a suitable buffer (HEPES buffer) or dilute nutrient broth, DNB (0.08 % Difco nutrient broth, 2 mM $CaCl_2 \cdot 2H_2O$, 3 mM $MgCl_2 \cdot 6H_2O$). The suspension is centrifuged again and the resulting pellet is finally resuspended in HEPES buffer or DNB. Calcium and Magnesium are usually added to the buffer to facilitate attachment between two negatively charged surfaces (Koval, 2006). In a study involving isolation of BALOs from sewage using enrichment method conducted by Koval & Hynes (1991), 200 mL of sewage was first supplemented with 50 mL DNB, incubated with shaking at 30 ^oC for 1 h and then centrifuged 3000 g for 10 min. The supernatant (20 mL) was then added to equal volume of washed prey cells. The enriched culture was then incubated at 30 ^oC for 48 h until appearance of bdelloplasts or fast moving attack phase of BALOs were observed under phase contrast microscope. The cultivation of BALOs then followed. In a similar way, Ruby (1992) used enrichment method to isolate BALOs from soil samples. In the study, 100 mg of soil was added to 50 mL of washed prey cells (10^9 or 10^{10} cells/mL) and incubated at 30 ^oC until presence of BALOs were confirmed by microscopy. The slurry was centrifuged at 2000 xg for 5 min and filtered through a 0.45 µm filter. The filtrate was diluted and cultured for BALOs isolation. In this current study, *Bdellovibrio* spp. were isolated from soil sample directly while sewage samples were enriched with prey cells before isolation. The enrichment of sewage was done by centrifuging the sewage sample at 5000 rpm for 15 min. The supernatant (10 mL aliquot) were then added to 20 mL of prey cells washed in HEPES buffer. The enriched culture was incubated at 30 ^oC for 7 days and observed for lysis which is monitored by reduction in turbidity.

After the separation of *Bdellovibrio* spp. from other environmental microbes or after the enrichment process, the cultivation of *Bdellovibrio* spp. on solid media is usually done by a method known as double layer agar plating technique (Stolp & Starr, 1963). The technique is similar to the

one used for the isolation of bacteriophage and relies on mixing diluted samples with a susceptible prey in soft agar (0.6 %). Most commonly used media for the isolation include YP medium (0.3 % yeast extract, 0.06 % peptone, pH 7.2), YP medium diluted tenfold (YP/10) and DNB agar medium (0.08 % nutrient broth amended with 3 mM MgCl2. $6H_2O$, 2 mM $CaCl_2$. $2H_2O$, 0.6 % agar for top agar and 1.9 % for bottom agar [pH 7.2 – 7.4]) . The growth of *Bdellovibrio* spp. is suspected when plaque forming units (PFU) are observed on culture plates after 48 h of incubation at 30 ^oC. The use of different dilutions of sample and diluted medium is to minimize or avoid overgrowth on *Bdellovibrio* spp. plaques by contaminating organisms present in the sample but not susceptible to attack by *Bdellovibrio* spp.

Another factor that is important to be considered while isolating *Bdellovibrio* spp. is the choice of prey. Unlike bacteriophages, the prey range of *Bdellovibrio* spp are not specific, however, *Bdellovibrio* spp. can attack wide range of Gram negative bacteria. The prey choice could also be determined based on the objectives of research. Furthermore, paracrystalline surface layers (S-layers) have been reported as barrier for predation in *B. bacteriovorus* (Koval and Hynes 1991). In our study, members of the Enterobacteriaceae most especially *Salmonella* spp. was observed as better option during the isolation process.

3.2. MATERIALS AND METHODS

3.3. Phenotypic characterization of *Bdellovibrio* spp.

*3.3.*1. Sample collection

Soil samples were collected at three different locations. The first site was at Centro de Biotecnología Genómica, Instituto Politécnico Nacional located in the city of Reynosa (26.069678N,-98.313108W and 26.069446N,-98.312902W) and the second site was in a Ranch located in Rio Bravo (25.984519N, -98.129108W), Mexico. The soil samples were collected with a clean hand trowel after removing about 20 - 25 mm top of soil and put into a sterile polythene bag. The sewage samples were collected from canal located around Universidad de Valle de Mexico (26.066685N,-98.277428W) and Boulevard Fundadores (26.089304N,-98.292479W) in Reynosa,

Tamaulipas, Mexico. The sewage sampling included area suspected to have biofilm such as sewage around plants and concrete surface in the sewage canal. The soil and sewage samples were transported to the laboratory for analysis and isolation of *Bdellovibrio* spp.

3.3.2. Determination of soil pH and electrical conductivity of soil samples.

The pH and electrical conductivity analysis was carried out on two soil samples obtained from Centro de Biotecnología Genómica, Instituto Politécnico Nacional (IPN), Reynosa, Mexico. Soil sample was suspended in 100 mL of deionized water and stirred for 5 min. The suspension was left on the bench overnight and stirred again afterwards. It was further left for 15 min and the liquid portion was transferred into a clean beaker. The pH and electrical conductivity of the soil sample was then measured using HI 991300TM pH/EC/TDS/Temperature meters (Hanna instruments, Rhode Island, USA) (Chaudari *et al*., 2014).

3.3.3. Preparation of bacterial prey

The bacterial preys used for preliminary isolation of the *Bdellovibrio* strains were *Klebsiella* sp. and *Salmonella* sp. donated by Biotechnology Institute (Autonomous University of Nuevo Leon [UANL as in Spanish] located in San Nicolás, Mexico) and Centro de Biotecnología Genómica, respectively. *Citrobacter freundii* CDBB-B-955 (ATCC 8090) was obtained from National Collection of Microbial Strains and Cell Cultures at the Research Center for Advanced Studies (CINVESTAV as in Spanish) of IPN located in México City, The different preys were cultured in Luria Bertani (LB) broth for 24 h at 37 ^{o}C. The bacterial preys were harvested by centrifugation, washed and re-suspended in 25 mM HEPES buffer (pH 7.2 - 7.4). The suspension was then used for the double layer agar plating technique and cultivation of *Bdellovibrio* strains in the liquid medium.

3.3.4. Isolation of *Bdellovibrio* spp.

Soil samples (100 g) were suspended in 100 ml of HEPES buffer and shaken for 1 h at 200 rpm. The suspension was centrifuged at 5,000 rpm for 15 min. The resulting supernatant was filtered serially using a 0.8 µm and 0.45 µm syringe filter (MF-Millipore^{TM} Membrane, Merck Millipore Ltd, Germany). The filtrate was serially diluted 10-fold (10^{-2} – 10^{-6}) in sterile HEPES buffer and plated on dilute nutrient broth (DNB) agar using double layer agar plating technique (Stolp & Starr,

1963; Jurkevitch, 2012). Briefly, 0.4 mL of the washed prey cells (~ 10^9 CFU/mL) suspended in HEPES buffer was mixed with 0.1 mL of sample filtrate in a sterile Falcon tube by pipetting. The mixture was then added to tube containing 4 mL of DNB top agar cooled to 45 ^oC, mixed by shaking gently and overlaid on DNB bottom agar in a standard 100 × 15 mm petri dish. After the top agar had solidified, the plates were incubated at 30 ^oC and plaque development on the DNB agar was monitored for 7 days. Plaques which emerge on DNB agar between 48 to 72 h, and progressively increase in size were taken to be potential *Bdellovibrio* plaques. The purification of plaques obtained was done by single plaque isolation technique (Jurkevitch, 2012) with slight modification. Briefly, single well-isolated plaques from plates were cut into 10 mL washed prey cells using pipette tip cut with a sterile scalpel and incubated at 30 ^oC until the suspension was clear. The lysate was then centrifuged at 5000 rpm for 15 min, filtered through 0.45 µm syringe filter, and cultured with wash prey bacteria cells using double layer agar plating technique. Sewage samples were enriched with washed bacterial prey cells prior to carrying out double layer agar plating technique as described above.

3.3.5. Storage of *Bdellovibrio* strains

For purpose of storage, the lysate obtained above was centrifuged twice at 2,000 rpm for 15 min at 4 ^oC. The supernatant was serially filtered through 0.80 µm, 0.65 µm and 0.45 µm syringe filter. At each stage of filtration, microscopic examination under Olympus U-TVO.35XC-2 (T2 Tokyo, Japan) light microscope was done to observe the presence of fast-moving *Bdellovibrio*. Also, cultivation of the filtrate on LB agar was carried out using spread plate technique in order to ascertain the total elimination of prey bacterial cell. The lysate and the pure *Bdellovibrio* strains obtained after serial filtration were stored in sterile glycerol at -80 ^oC for further study and long-term storage.

3.3.6. Phenotypic characterization of *Bdellovibrio* strains

The phenotypic characterization of the isolated *Bdellovibrio* strains was based on plaque morphology, microscopy techniques and prey range analysis. Plaques formed by *Bdellovibrio* strains are expected to develop between 48 – 72 h, however, some plaques which appeared after

24 h and increased in size with increased days of incubation were analyzed for the presence of *Bdellovibrio* strains. The two microscopy techniques employed in this study were light and atomic force microscopy. The light microscope was used to initially detect the *Bdellovibrio* strains based on characteristic high motility and small size compared to the prey cells. The atomic force microscopy (AFM) was done at the Department of Cellular Biology and Genetics, Universidad Autonomas de Nuevo Leon (UANL), San Nicolas, Mexico. *B. bacteriovorus* strain SSB218315 and *Salmonella* sp. (prey) were co-cultured in HEPES buffer in a shaker incubator at 30 ^{o}C for 48 h (when the lytic activity begins). The lysates were then transported to UANL for AFM. Then 10 µL of lysate from the co-culture in the HEPES buffer was deposited on cleaved mica and allowed to air-dry. The bacteria samples were observed using an NT-MDT NTEGRA Prima AFM at room temperature, with a RTESPA probe (Bruker corporation, China) of spring constant k = 40 N/m in intermittent contact mode. Images of height, deflection and phase were obtained; 20 x 20, 10 x 10, and 5 x 5 µm^2 image sizes were captured systematically for each sample at three different regions at least. They were analyzed with WSxM software to observe the morphological aspect of the bacteria (Nunez *et al*. 2003; Horcas *et al*. 2007).

3.3.7. Determination of lytic activity of *Bdellovibrio* strains.

A single pure plaque was cut into 5 mL suspension of bacterial prey cells already washed with HEPES buffer using pipette tip cut with a sterile scalpel. The culture was incubated at 30 ^{o}C and monitored for prey lysis. Microscopic examination was carried out to determine the presence of highly motile *Bdellovibrio* strains. The clear HEPES buffer culture obtained from the prey lysis was centrifuged three times at 3,000 rpm for 15 min (Schwudke *et al*. 2001). The resultant supernatant (the lysate) was further used to inoculate large volume of susceptible bacterial prey already washed with HEPES buffer. The initial optical density of the prey was read at 600 nM by using optizen POP spectrophotometer (Mecasys Co., Ltd, Daejeon, Korea) and the progressive reduction in turbidity with time was monitored to determine the lytic activity of the *Bdellovibrio* strains.

3.3.8. Prey range analysis

The prey range analysis for two strains (SKB1291214 and SSB218315) was carried out using double layer agar plating technique as described above with thirty-six bacterial isolates including twenty-one referenced bacterial isolates obtained from the National Collection of Microbial Strains and Cell Cultures at the, Research Center for Advanced Studies (CINVESTAV as in Spanish) of IPN located in México City, México and fifteen laboratory bacterial strains obtained from Biotechnology Institute of UANL and Centro de Biotecnología Genómica, Instituto Politécnico Nacional (IPN), Reynosa, Mexico. The reference bacterial strains were cultured as recommended by the culture collection center while the laboratory strains were cultured in LB medium for 24 h at 37 ^{o}C. The experiment was carried out in triplicates and plaque formation was monitored for at least 7 days. Prey range determination for *Bdellovibrio* strains SKUVM1 and SCRB3 was carried by cocultivation of predator and prey in a microtitre plate, and the result was obtained using iMark™ Microplate Absorbance Reader (Hercules, California, United States). The experiment was carried out in triplicates and bacterial lysis was monitored for at least 7 days.

3.3.9. Statistical analysis

Each experiment and control for determination of lytic activity was carried out in triplicates with *Klebsiella* sp. and *Salmonella* sp. preys suspended in HEPES buffer without *Bdellovibrio* strains serving as control. The transformation of mean values of optical density was done using square root for variance normalization. The transformed mean values of optical density (on y-axis) was plotted against time (on x-axis). Statistical analysis was performed using Excel© for Windows©, 2013. Student's t-test was used to compare the mean values of the two groups and $P<0.05$ was used as the indicator of significant difference.

3.4. Molecular characterization of *Bdellovibrio* spp.

3.4.1. Amplification of 16S rDNA and host interaction (*hit*) locus

For the detection of *Bdellovibrio* strains, a clear lysate was centrifuged three times at 3,000 rpm for 15 min to remove residual prey cells (Schuwdke *et al*. 2001). The final resultant supernatant was centrifuged at 27,000 x g for 20 min. The presence of few residual prey bacterial cells even

after centrifugation was observed in the present study and reported by Parker & Grove, (1970) and Schuwdke *et al*. (2001). Therefore for purification and amplification of considerable length of the 16S rRNA gene (1493 bp), a large volume of lysates from prey-predator co-culture was centrifuged twice at 2,000 rpm for 15 min at 4 ^{o}C. The supernatant was serially filtered through 0.80 µm, 0.65 µm and 0.45 µm syringe filter. At each stage of filtration, microscopic examination to observe the presence of fast-moving *Bdellovibrio* as well as cultivation of the filtrate on LB agar using spread plate technique to ascertain total elimination of prey bacterial cell was done. In addition, double layer agar plating technique was carried out at each stage of filtration to ascertain the presence of plaque-forming *Bdellovibrio* strains. Finally, the filtrate obtained was centrifuged at 27,000 x g for 20 min in an Eppendorf tube. The pellet obtained from the two processes were washed with sterile water, centrifuged and the resulting pellet was used for DNA extraction.

3.4.2. Genomic DNA extraction from *Bdellovibrio* spp.

DNA extraction was done using Promega Wizard® Genomic DNA purification kit (Madison, Wisconsin, United States) according to manufacturer's instructions. The *Bdellovibrio* pellet in each Eppendeorf tube was resuspended in 600 µL of nuclei lysis solution (supplied) by pipetting gently. The suspension was incubated for 5 min at 80 ^{o}C in a thermomixer (Hamburg, Germany) without shaking and the tubes were then allowed to cool to room temperature. After, 3 µl of RNase solution (supplied) was added to each tube, mixed gently by pipetting and incubated at 37 ^{o}C for 1 h in the thermomixer. Thereafter, the tubes were removed from the thermomixer and allowed to cool to room temperature before adding 200 µL of protein precipitation solution (supplied). The tubes were vortexed and incubated on ice for 5 min before centrifuging at 13,000 rpm for 3 min. The supernatant from each tube was transferred into a clean tube containing 600 µL of room temperature molecular biology grade isopropanol (Sigma; St. Louis, Missouri, United States). The tubes were shaken by inverting the tubes gently until DNA strands were visible. The tubes were centrifuged and for 2 min at 13, 000 rpm and the supernatant in each tube was discarded carefully. The DNA pellet was washed by adding 600 µL of 70 % room temperature molecular biology grade ethanol (Sigma; St. Louis, Missouri, United States) into each tube. The tubes were again shaken by inverting gently and centrifuged at 3, 000 rpm for 2 min. The ethanol in each tube was carefully

aspirated using pipette and the tubes were carefully inverted on clean absorbent paper. The tubes were further left for 15 min to air-dry the pellet. Finally, 50 µL rehydration solution (supplied) was added to each tubes and incubated for 1 h at 65 ^{o}C in a thermomixer. For confirmation of successful DNA extraction, 2 µL of the resulting product of DNA extraction was detected by gel electrophoresis using 1 % w/v agarose and run in 1X TAE buffer pH 8.3 for 30 min at 90 Volts. Thereafter, the electrophoresis gel was visualized in Kodak electrophoresis documentation and analysis system loaded with GLogic 112 camera and Kodak bioinformatics program Ds 1D. The DNA samples that were successfully extracted were stored at -20 ^{o}C for further use.

The genomic DNA concentration was determined with Nanodrop™ (Thermoscientific, Waltham, Massachusetts, United States) at 260 nm wavelength using rehydration solution as reference blank. The purity of the genomic DNA was calculated by the ratio of the absorbance at 260 nm and 280 nm. A pure DNA which have less contaminants such as protein is expected to have A_{260}/A_{280} of 1.8-2.0.

3.4.3. Amplification of 16S rRNA gene and host interaction (*hit*) locus by Polymerase Chain Reaction (PCR)

The primer sets and PCR conditions used for the PCR amplification of *Bdellovibrio* 16S rRNA gene and *hit* locus were as shown in Table 3.1. All PCR reactions were carried out separately in a final volume of 25 µL in 0.2 mL PCR tubes. The reaction mixtures consisted of 30 ng/uL of DNA template and master mix containing 1 X colourless Gotaq® Reaction Buffer (with 1 mM $MgCl_2$), 10 mM dntps, 0.4 µM for each forward and reverse primer and 0.2 units of GoTaq® DNA polymerase. Sterile milliQ water was added to make a final volume of 25 µL. The genomic DNA of reference strain *B. bacteriovorus* HD100 obtained from Prof. Edouard Jurkevitch of the Hebrew University of Jerusalem and sterile milliQ water served as positive and negative control respectively. The PCR products were detected by gel electrophoresis using 1 % w/v agarose (Sigma; St. Louis, Missouri, United States) and run in 1X TAE buffer pH 8.3 for 1 h at 90 Volts. The gel was visualized in Kodak electrophoresis documentation and analysis system loaded with GLogic 112 camera. The gel was analyzed by comparing the corresponding band for the amplified

16S rRNA gene and *hit* locus with the positive control and 100 bp DNA ladder (Promega® Madison, Wisconsin, United States).

Table 3. 1. List of Primers and PCR conditions for the amplification and sequencing of the 16S rRNA gene and *hit* locus

Primer Name	Primer (Size)	PCR conditions	Reference
16S rDNA I	BbsF216: 5' TTTCGCTCTAAGATGAGTCCGCGT 3' BbsR707: 5' TTCGCCTCCGGTATTCCTGTTGAT 3' (492 bp)	Initial Denaturation: 95^{o}C for 5 mins Denaturation: 95^{o}C for 30 secs Annealing: 60^{o}C for 30 secs (58.5^{o}C for the *hit* locus) Extension: 72^{o}C for 40 secs Final Extension: 72^{o}C for 10 mins Hold: 4^{o}C forever	Van Essche *et al*., 2009
hit locus	BdhitF: 5' TCTAGACAGATGGGATTACTG 3' BdhitR: 5' GAATTCTGGCATCAACAGC 3' (959 bp)		This study http://*insilico*.ehu.es/PCR/Amplify. Online tool of University of the Basque country
16S rDNA II	BdelloF: 5' AGAGTTTGATTCTGGCTCAGA 3' BdelloR: 5' AGGTGATCCAGCCGCAGGTTC 3' (1493 bp)	Initial Denaturation: 95^{o}C for 5 mins Denaturation: 95^{o}C for 30 secs Annealing: 62^{o}C for 30 secs Extension: 72^{o}C for 40 secs Final Extension: 72^{o}C for 10 mins Hold: 4^{o}C forever	This study http://*insilico*.ehu.es/PCR/Amplify. Online tool of University of the Basque country.

3.4.4. Purification of PCR products

The amplified DNA fragment was purified using QIAquick ® Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Each PCR product (20 µL) was resolved by gel electrophoresis using 1 % w/v agarose (Sigma; St. Louis, Missouri, United States) and run in 1X TAE buffer pH 8.3 for 1 h at 90 Volts. The DNA fragment was excised from the gel using clean, sharp scalpel (Extra gel around the DNA fragment were cut off to reduce the size of the gel). The gel was weighed in a clean 1.5 mL centrifuge tube, and buffer QG (supplied) was added at the ratio of 3 volume buffer QG to 1 volume of gel (100 mg ~ 100 µl). The sample was incubated at 50 ^{o}C for 10 min and in order to ensure complete dissolution of gel in the buffer QG, the tube was vortexed every 2–3 min during the incubation. Thereafter, one gel volume (100 mg to 100 µL) of isopropanol (Sigma; St. Louis, Missouri, United States) was added to the sample. The sample was transferred into a QIAquick spin column (for DNA binding), placed in a 2 mL centrifuge tube and centrifuged for 1 min at 13, 000 rpm. The flow-through was discarded and the QIAquick spin column was placed back into the 2 mL centrifuge tube. Buffer PE (0.75 mL, supplied) was added to the tube (to wash the DNA) and centrifuged at for 1 min at 13, 000 rpm. The flow-through was discard and the QIAquick spin column inserted into the 2 mL centrifuge tube was again centrifuged for 1 min at 13, 000 rpm to remove traces of the buffer PE. The flow-through was discarded and the QIAquick spin column was placed in a clean 1.5 mL centrifuge tube to elute the DNA. For the DNA elution, 30 uL of sterile milliQ wáter was added to the center of the QIAquick spin column, and centrifuged at 13, 000 rpm for 1 min after allowing the column to stand for 1 min. The purified eluted DNA was used for cloning and direct sequencing. The remaining DNA was stored at -20 ^{o}C.

3.4.5. Cloning of the amplified 16S rRNA gene fragment

The cloning process was done to obtain identical copies of the 16S rRNA gene of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315. The purified PCR product of 16S rDNA was cloned using PGEM®-T Easy vector (Promega® Madison, Wisconsin, United States) (Figure 3. 1). The major steps involved in the cloning process were obtaining PCR product, ligation (inserting or joining) of PCR product into a suitable vector, transformation (incorporating the ligated

vector into a suitable competent host), selection of successful transformants (bacterial host that have the vector-PCR fragment), cultivation of successful and transformants in a suitable medium, extraction and sequencing of plasmids.

In this present study, the PGEM®-T Easy vector was centrifuged to collect the content at the bottom of the tube. The ligation reaction was performed in a ratio 1:3 of vector to insert. The ligation reaction set up was set up as below:

Rapid ligation buffer (2X)	5.0 µL (1X)
PGEM®-T Easy vector	50 ng
PCR product	20 ng
T4 DNA Ligase	3 units/µL
Deionized water to a final volume of	10

The reaction was mixed by pipetting and incubated overnight at 4 °C. For the transformation process, the ligation reaction was centrifuged briefly and 5 µL of each ligation reaction was added to sterile 1.5 mL tube on ice. Then 100 µL of competent *E. coli* DH5α cells was added to the ligation reaction tube. The tube was gently flicked and incubated on ice for 20 min. The *E. coli* DH5α cells in the ligation reaction tube were heat shocked at 42 °C in a thermomixer for 1 min, and the tube was immediately returned to ice for 2 min. Thereafter, 950 µL of LB broth was added to the ligation reaction transformations, and incubated at 37 °C for 2 h in a thermomixer at 550 rpm. After the incubation period, the cell culture was centrifuged at 5000 rpm for 5 min and resuspended in 100 µL of LB broth. The resuspended transformation culture, 100 µL of IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 50 µL of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was spread on LB agar incorporated with ampicillin using sterile glass spreader. The plates were incubated overnight at 37 °C. After incubation, the plates were examined and white colonies which are the successful transformants were cultured in LB broth supplemented with ampicillin. The blue colonies observed on the plates were bacterial hosts that were not successful for the transformation process (blue-white screening).

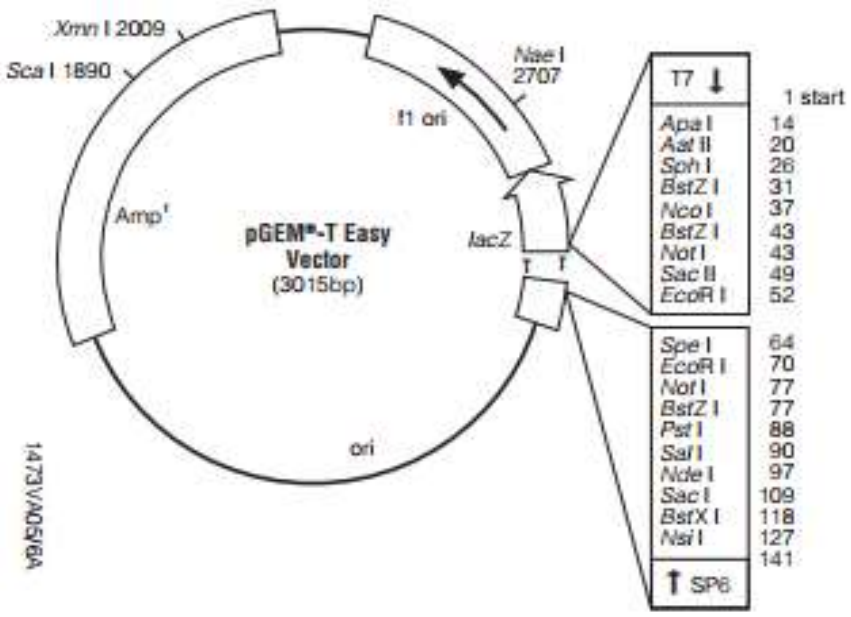


Figure 3. 1. Composition of the PGEM®-T Easy vector used for the cloning of the amplified 16S rRNA gene. (PROMEGA Inc).

3.4.6. Plasmid extraction from successful transformants

Plasmid extraction was done using QIAprep®Spin Miniprep Kit (Qiagen, Hilden, Germany). An overnight LB culture (1 mL) of transformants (white colonies) was centrifuged at 13,000 rpm for 3 min at room temperature. The pellet obtained was resuspended in 250 µL buffer P1 (supplied). Thereafter, 250 µL of buffer P2 was added and the mixture was mixed thoroughly by inverting the tube 4-6 times until the solution became clear. Buffer N3 (350 µL) was added, and the tube mixed immediately and thoroughly by inverting the tube 4-6 times. The tube was centrifuged at 13, 000 rpm for 10 min, and the supernatant was transferred to a QIAprep spin column by pipetting. The spin column was centrifuged for 1 min at 13,000 rpm and the flow through was discarded. Buffer PB (0.5 mL; supplied) was then added and the spin column was centrifuged at 13, 000 rpm for 1 min. The flow-through was discarded. The plasmid was washed by adding 0.75 mL of buffer PE (supplied) and centrifuging at 13,000 rpm for 1 min. The flow-through was discarded and the spin column was centrifuged again at 13,000 rpm for 1 min to remove residual wash buffer. The QIAprep spin column was placed in a clean 1.5 mL centrifuge tube to elute the plasmid. For the plasmid elution, 30 µL of sterile milliQ wáter was added to the center of the QIAprep spin column and

centrifuged at 13, 000 rpm for 1 min after allowing the column to stand for 1 min. For confirmation of successful plasmid extraction, 2 µL of the resulting product of plasmid extraction was detected by gel electrophoresis using 1 % w/v agarose and run in 1X TAE buffer pH 8.3 for 1 h at 90 Volts along with 1 Kb ladder. Thereafter, the electrophoresis gel was visualized in Kodak electrophoresis documentation and analysis system loaded with GLogic 112 camera and Kodak bioinformatics program Ds 1D. The eluted plasmid was sequenced by Eurofins MWG Operon© LLC company (www.operon.com; 2211 Seminole Drive Huntsville, Alabama 35805, USA) using primers; T7: 5' TAATACGACTCACTATAGGG 3' and M13: 5' CAGGAAACAGCTATGAC 3'.

3.4.7. Sequencing of PCR product, assembly of sequences and BLAST search analysis in Nacional Center for Biotechnology Information (NCBI) and ribosomal database Project (RDP)

The sequencing of the purified PCR products was done by Eurofins MWG Operon© LLC company (www.operon.com; 2211 Seminole Drive Huntsville, Alabama 35805, USA) using Sanger method. The chromatogram result of sequencing from Eurofins Eurofins MWG Operon© LLC Company was opened, edited and cleaned using FinchTV software. The Sequence assembly and generation of a consensus sequence for the 16S rDNA and *hit* locus was done using Lasergene program Seqman® software (DNAstar Inc., Madison, WI). The consensus sequences were used for homology or similarity BLAST (Blast local alignment Search tool) searches with online Blastn bioinformatic program developed by Nacional Center for Biotechnology Information (NCBI) (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) and ribosomal database Project (RDP) (*http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp*). Phylogenetic tree was constructed with Neighbor Joining (NJ) algorithm using the pairwise distances (Kimura two-parameter model) metric to recover their clustering pattern. Bootstrap values were calculated to test the robustness of interior node support and were obtained by conducting 1,000 pseudoreplicates using MEGA© 6.0 software (Tamura *et al*., 2013).

3.4.8. Characterization of *Bdellovibrio* spp. using Amplified rDNA (Ribosomal DNA) Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis is a technique used to distinguish microbes based on the PCR amplification of 16S rDNA, followed by restriction digestion using different restriction enzyme or endonucleases that cut the amplified PCR product of the 16S rDNA at a specific nucleotide sequence. The digested PCR products are then analysed using gel electrophoresis, and different restriction patterns observed represent different ribotypes (species). The flow chart for carrying out ARDRA illustrated below (Figure 3. 2.). The ARDRA was employed to complement the result obtained from the 16S rRNA gene sequencing and to further differentiate *Bdellovibrio* spp. Strains SKB1291214 and SSB218315. In this study, 1493 bp fragment of the 16S rDNA for *Bdellovibrio* sp. SKB1291214, *B. bacteriovorus* SSB218315 and *B. bacteriovorus* HD100 was amplified using the primers and PCR conditions described in section 3.1.3.3 above. The amplified PCR products were purified as earlier described and digested with the following restriction enzymes: *BamHI*, *EcoRI*, *HindIII*, *XbaI* and *XhoI* according to the restriction reaction set up below:

Sterile milliQ water	9.5 µL
Restriction enzyme buffer (10 X)	2 µL
Bovine Serum Albumin (BSA) Acetylated (1 mg/mL)	0.5 µL
DNA (0.2 – 1 µg)	7 µL
Restriction enzyme (2-10 U)	1 µL
Final volume	20 µL

The digestion mixture was incubated for 4 h at 37 ºC in thin-wall tubes. Restriction fragment patterns were analyzed by electrophoresis on 1% w/v agarose gel. The restriction pattern generated was visually examined and analysed.

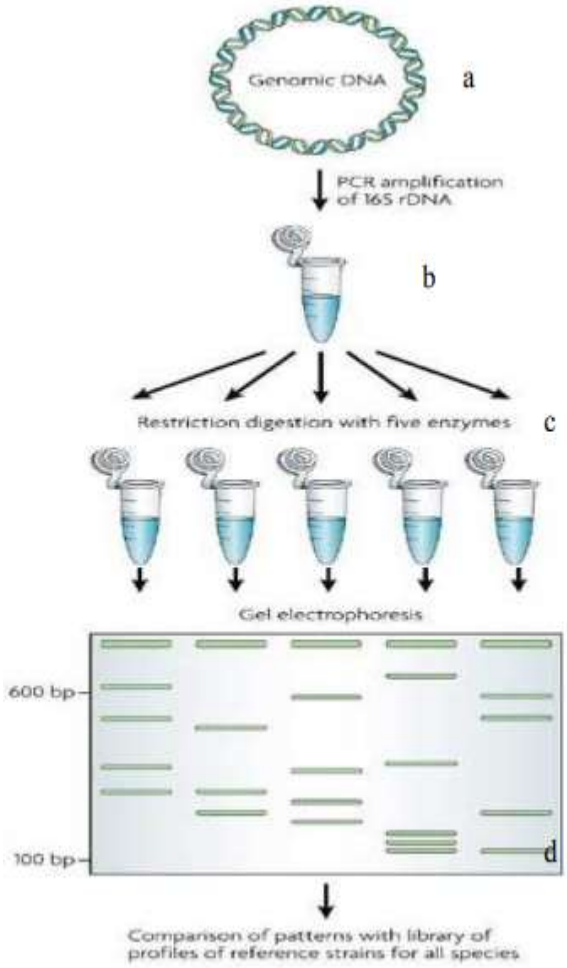


Figure 3. 2. Steps of Amplified rDNA Restriction **Analysis** (a: Genomic DNA extraction, b: PCR reaction for specific region, c: restriction digestion, d: gel electrophoresis) (Dijkshoorn *et al*., 2007).

RESULTS

3.5. Phenotypic Characterization of *Bdellovibrio* spp.

3.5.1. Isolation of *Bdellovibrio* spp. from soil and sewage in Reynosa, Tamaulipas, Mexico.

Several samples were collected at different sites in Reynosa, Tamaulipas, Mexico. The location of the soil and sewage where *Bdellovibrio* spp. were successfully isolated is shown in Table 3. 2. The direct method was used for the isolation of *Bdellovibrio* spp. from the soil samples while the enrichment method was used for the isolation from sewage samples. The characteristics of the soil samples showed that they have good agricultural value while the sewage was polluted with organic wastes (Table 3. 2). A total of five predatory bacteria strains including four strains belonging to the Genus *Bdellovibrio* and a strain belonging to the Genus *Bacteriovorax* were isolated. Two different strains of *Bdellovibrio* designated SKB1291214 and SSB218315 were isolated from soil under a banana (*Musa paradisiaca* L) plant at a neighborhood from the city of Reynosa, Mexico (IPN), using DNB agar with plaque development observed within 2-7 days on *Klebsiella* sp. and *Salmonella* sp. preys, respectively. Later, another strain designated SKUVM1 was obtained from sewage in Reynosa using *Klebsiella* sp. while *Bdellovibrio* strain SCRB3 was isolated from soil in a Ranch located in Rio Bravo, Tamaulipas, Mexico (about 23 km away from Reynosa) using *Citrobacter freundii* CDBB-B-955 (ATCC 8090). Another strain designated SSFD2 belonging to the Genus *Bacteriovorax* was isolated from sewage sample in Reynosa using *Salmonella* sp. The photograph image of Plaque Forming Units (PFU) formed by *Bdellovibrio* sp. SKB1291214 after 48 h and 72 h when cultured with *Klebiella* sp. at 30 ^{o}C on DNB agar is shown in Figure 3. 3 and 3. 4. The isolated *Bdellovibrio* strains formed irregular and clear plaques that expanded during the period of incubation. The light microscopy examination revealed the isolated *Bdellovibrio* strains as highly motile, rod (comma) shaped Gram-negative bacteria.

Table 3. 2. The sampling location in Reynosa and Rio Bravo, Tamaulipas, Mexico where soil and sewage samples were collected for the isolation of *Bdellovibrio* spp.

SAMPLE	LOCATION	DESCRIPTION	pH	EC
A	CBG, Reynosa (26.069678N,-98.313108W)	Dark-brown moist loamy soil	7.30	0.26
B.	CBG, Reynosa (26.069446N,-98.312902W)	Dark-brown moist loamy soil	7.38	0.16
C.	Ranch in Rio Bravo (25.984519N,-98.129108W)	Dark-brown moist loamy soil	ND	ND
D.	UVM area, Reynosa (26.066685N,-98.277428W)	Polluted sewage	ND	ND
E.	Boulevard Fundadores, Reynosa (26.089304N,-98.292479W)	Polluted sewage	ND	ND

ND: Not determined; Rio Bravo is a town that is 23 kilometers away from Reynosa; UVM: Universidad de Valle de Mexico; CBG: Centro de Biotecnología Genómica

Figure 3. 3. The photographic image of the plaque forming unit (PFU) produced by *Bdellovibrio* sp. SKB1291214. Plaque was observed after 48 h when cultured with *Klebsiella* sp. at 30 ^{o}C on DNB agar plate.

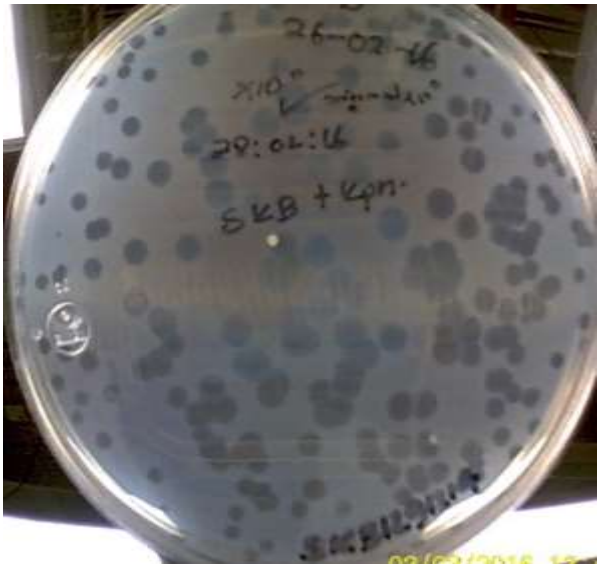


Figure 3. 4. The photographic image showing the increase in the plaque forming unit (PFU) produced by *Bdellovibrio* sp. SKB1291214. The plaques kept expanding even after 72 h.

3.5.2. Characterization of *Bdellovibrio* spp. under Atomic Force Microscope (AFM)

The Figure 3. 5. below showed the different features that characterized the life cycle of *Bdellovibrio* spp. when *B. bacteriovorus* SSB218315 was examined under atomic force microscopy. The AFM revealed clearly two kinds of bacteria, the small comma-shaped *B. bacteriovorus* (predator indicated with red arrow) and the bigger long rod-shaped *Salmonella* sp (prey indicated with white arrow). A structure resembling the Bdelloplast usually formed by *Bdellovibrio* spp. during the intraperiplasmic invasion was also observed (indicated by black arrow). The enlarged image of the comma-shaped *B. bacteriovorus* SSB218315 was also viewed under AFM (Figure 3. 6) The image showing the attachment of *B. bacteriovorus* SSB218315 to the *Salmonella* sp. prey was also observed (Figure 3. 7) while another image showed the clustering of the *Bdellovibrio* strains in a fashion that perhaps depict a prey cell that was just lysed to release *Bdellovibrio* progenies (Figure 3. 8).

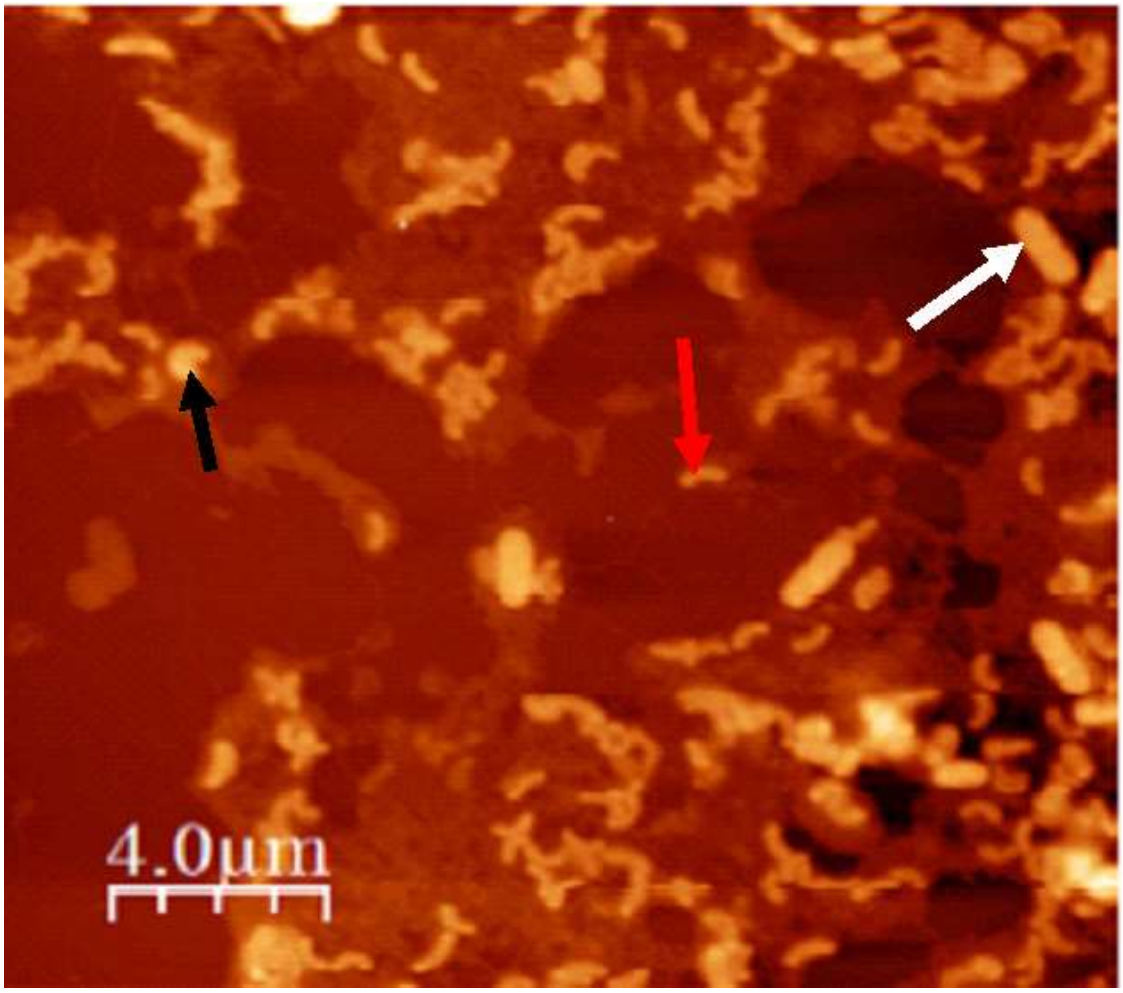


Figure 3. 5. Atomic force microscopy showing different images that characterize the life cycle of *Bdellovibrio* spp when *B. bacteriovorus* SSB218315 was co-cultured with *Salmonella* sp. The long rod shaped *Salmonella* sp. (indicated by the white arrow), the small “comma-shaped or vibroid” *B. bacteriovorus* SSB218315 (indicated by the red arrow) and the Bdelloplast structure (indicated by the black arrow).

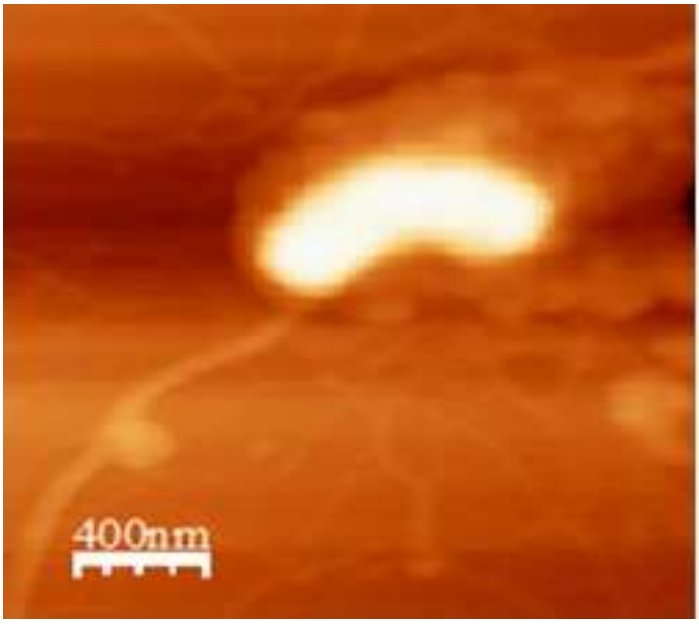


Figure 3. 6. Atomic force microscope showing enlarged image of “comma-shaped or vibroid” *B. bacteriovorus* SSB218315

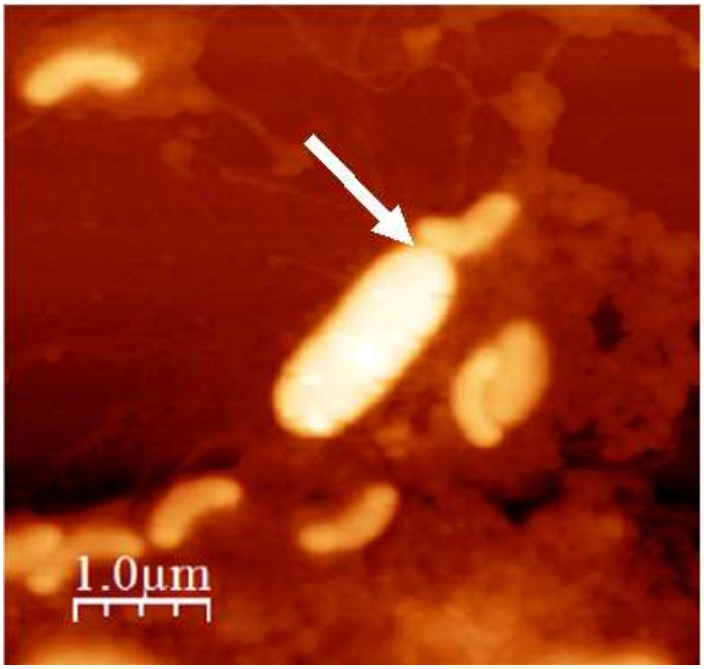


Figure 3. 7. Atomic force microscope showing the image of “comma-shaped or vibroid” *B. bacteriovorus* SSB218315 attached to the long rod-shaped *Salmonella* sp (indicated by the white arrow).

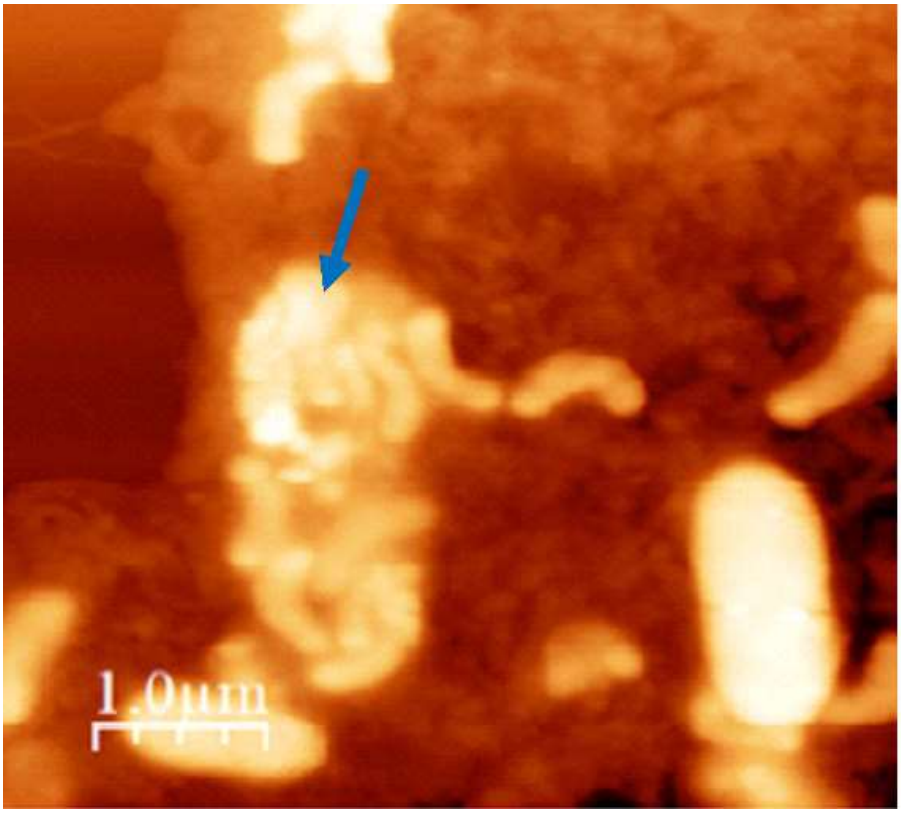


Figure 3. 8. Atomic force microscope showing a structure resembling lysed prey cells with the release of *Bdellovibrio* progenies (indicated by the blue arrow).

3.5.3. Determination of the lytic activity of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 in liquid medium

Bdellovibrio sp. SKB1291214 was able to attack and lyse the *Klebsiella* sp. in liquid medium. This was evident with the reduction in optical density [(1.07 to 0.26) before statistical square root transformation] within 72 h (Figure 3. 9). Similarly, *B. bacteriovorus* SSB218315 lysed the *Salmonella* sp. in liquid medium reducing the optical density from 0.98 to 0.15 [values obtained before statistical square root transformation (Figure 3. 10)]. The statistical analysis of the mean optical density values of the experimental (0.90 ± 0.28 SD) when *Klebsiella* sp. was infected with *Bdellovibrio* sp. SKB1291214 was significantly different in comparison with the mean optical density values of the control (1.22 ± 0.09 SD) as determined by student's t-test ($t = 0.007$, $P < 0.05$). In a similar way, there was significant difference ($t = 0.01$, $P < 0.05$) using student's t-test to compare

mean optical density values of the experimental (0.65 ± 0.26 SD) and control (0.87 ± 0.05 SD) when *Salmonella* sp. was infected with *B. bacteriovorus* SSB218315.

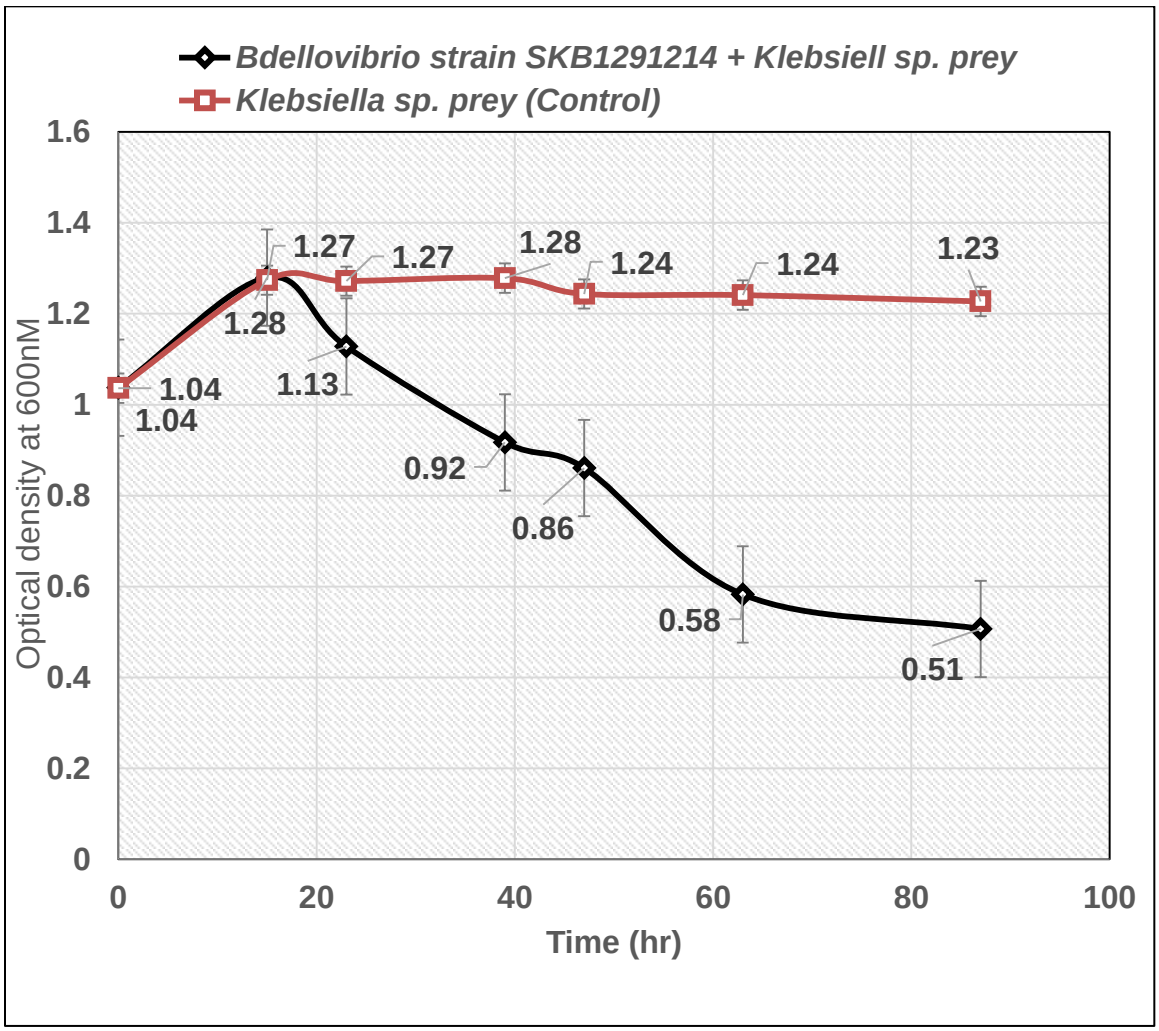


Figure 3. 9. The lytic activity of *Bdellovibrio* sp. SKB1291214 (1.22×10^6 $PFUml^{-1}$) when *Klebsiella* sp. was infected. The graph shows mean values of optical density (y-axis) against time (x-axis) with error bars showing standard error. R^2 = 0.81 (for experimental) and 0.16 (for control). *t* = 0.01, P < 0.05.

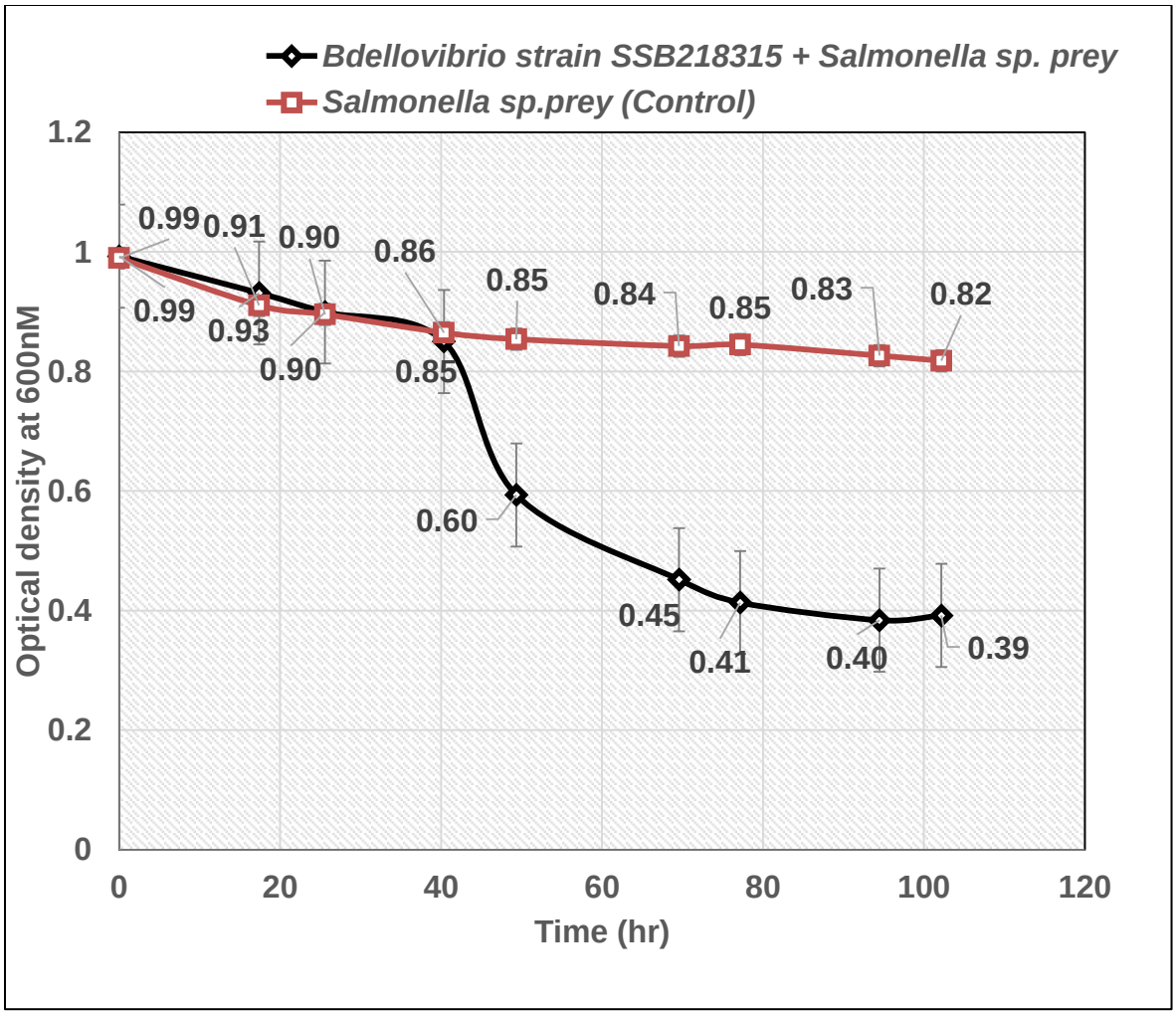


Figure 3. 10. The lytic activity of *B. bacteriovorus* SSB218315 (8.62×10^5 $PFUml^{-1}$) when *Salmonella* sp. was infected. The graph shows mean values of optical density (y-axis) against time (x-axis) with error bars showing standard error. $R^2 = 0.91$ (for experimental) and 0.83 (for control). $t = 0.02$, $P < 0.05$.

3.5.4. Determination of Prey range

Bdellovibrio sp. SKB1291214 was able to form plaque on lawns of 13 out of 36 (36.11 %) bacterial isolates considered for prey range analysis. It preyed only upon some members of the family Enterobacteriaceae except *Proteus mirabilis* CDBB-B-1343 (ATCC 21100), *S. marcescens* and some strains of *E. coli* [(*Escherichia coli* CDBB-B-1107 (ATCC 8739), *Escherichia coli* 2 and *Escherichia coli* DH5α]. Meanwhile, *B. bacteriovorus* SSB218315 was able to prey upon 22 (61.11 %) bacterial isolates which include family Enterobacteriaceae except *Citrobacter freundii* CDBB-B-

955 (ATCC 8090) and *Escherichia coli* DH5α. It also preyed upon members of the genus *Pseudomonas*, *Vibrio cholerae CDBB-B 1159 (ATCC 39540)* and *Alcaligenes* sp. CDBB-B-17 (ATCC 27066). *Bdellovibrio* sp. SKB1291214 and SSB218315 were unable to prey upon *Rhizobium leguminosarum* (CDBB-B- 1885), *Agrobacterium tumefaciens* (CDBB-B-1042), and *Pseudomonas syringae patovar aceris* (ATCC 10853). *Bdellovibrio* spp. SKUVM1 and SCRB3 exhibited same prey range pattern. They prey upon members of the family Enterobacteriaceae, the genus *Pseudomonas* and *Stenotrophomonas, Vibrio cholerae CDBB-B 1159 (ATCC 39540)* and *Alcaligenes* sp. CDBB-B-17 (ATCC 27066). Furthermore, *Bdellovibrio* sp. SKUVM1 and SCRB3 showed the ability to lyse *Staphylococcus epidermidis*, a Gram-positive bacterium by reducing the optical density of the prey cells when cocultured in a microtiter plate. All *Bdellovibrio* strains did not prey upon strains of *Staphylococcus aureus*, *Bacillus thurigiensis*, and *Bacillus cereus* (Table 3.3).

Table 3. 3. Prey range analysis of *Bdellovibrio* strains SKB1291214, SSB218315, SKUVM1 and SCRB3 on thirty-six (36) bacterial isolates.

S/No	Bacterial Prey	SKB1291214 (36.11 %)	SSB218315 (61.11 %)	SKUVM1 (77.7 %)	SCRB3 (77.7 %)
1	*Klebsiella oxytoca* B-968 (ATCC 13182)[1]	+	+	+	+
2.	*Klebsiella pneumoniae subspecie pneumonia* B-969 (ATCC 13883)[1]	+	+	+	+
3.	[4]*Klebsiella* sp.[2]	+	+	+	+
4.	*Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251)[1]	+	+	+	+
5.	[5]*Salmonella* sp A[3]	+	+	+	+
6.	*Salmonella* sp B[3]	+	+	+	+
7.	*Salmonella* sp D[3]	+	+	+	+
8.	*Pseudomonas aeruginosa* CDBB-B-1021 (ATCC 27853)[1]	-	+	+	+
9.	*Pseudomonas putida* CDBB-B-93 (ATCC 795)[1]	-	+	+	+
10.	*Pseudomonas fluorescens CDBB-B*-1243 (ATCC 13525)[1]	-	+	+	+
11.	*Enterobacter aerogenes* CDBB B-958 (ATCC 13048)[1]	+	+	+	+
12.	*Serratia marcescens* CDBB-B-1014 (ATCC 14756)[1]	-	+	+	+
13.	*Vibrio cholerae CDBB-B 1159 (ATCC 39540)*[1]	-	+	+	+
14.	*Staphylococcus aureus* subsp *aureus* CDBB-B-1001 (ATCC 6538)[1]	-	-	-	-
15.	*Staphylococcus aureus* AR2[3]	-	-	-	-
16.	*Staphylococcus aureus B*[3]	-	-	-	-
17.	*Staphylococcus epidermidis* CDBB-B-1012 (ATCC 12228)[1]	-	-	+	+
18.	*Bacillus thuringiensis* CDBB-B-26 (ATCC 13366)[1]	-	-	-	-
19.	*Bacillus cereus* CDBB-B-949 (ATCC 6464)[1]	-	-	-	-
20.	[6]*Citrobacter freundii* CDBB-B-955 (ATCC 8090)[1]	+	-	+	+
21.	*Alcaligenes* sp. CDBB-B-17 (ATCC 27066)[1]	-	+	+	+
22.	*Escherichia coli* CDBB-B-1107 (ATCC 8739)[1]	-	+	+	+
23.	*Escherichia coli* 5A[3]	+	+	+	+
24.	*Escherichia coli* 5B[3]	+	+	+	+
25.	*Escherichia coli* 2[3]	-	+	+	+
26.	*Escherichia coli* 4[3]	+	+	+	+
27.	*Escherichia coli* 3B[3]	+	+	+	+
28.	*Escherichia coli* DH5α[1]	-	-	+	+
29.	*Proteus mirabilis* CDBB-B-1343 (ATCC 21100)[1]	-	+	+	+
30.	*Stenotrophomonas sp* C18414[3]	-	-	+	+
31.	*Stenotrophomonas sp* A23414[3]	-	-	+	+
32.	*Stenotrophomonas sp* CAZ[3]	-	-	+	+
33.	*Pseudomonas sp* DTB[3]	-	+	+	+
34.	*Rhizobium leguminosarum* (CDBB-B- 1885)[1]	-	-	ND	ND
35.	*Agrobacterium tumefaciens* (CDBB-B-1042)[1]	-	-	ND	ND
36.	*Pseudomonas syringae patovar aceris* (ATCC 10853)[1]	-	-	ND	ND
	N	**13**	**22**	**28**	**28**

N: number of bacteria preyed upon [1]Reference bacterial isolates obtained from National Collection of Microbial Strains and Cell Culture of CINVESTAV., [2]Laboratory bacterial strains obtained from Biotechnology Institute of UANL., [3]laboratory bacterial strains obtained from Center for Genomic Biotechnology (IPN), [4] positive control for *Bdellovibrio* strain SKB1291214 and SKUVM1, [5] positive control for *Bdellovibrio* strain SSB218315, [6] positive control for *Bdellovibrio* strain SCRB3.

3.6. Molecular characterization of *Bdellovibrio* spp.

3.6.1. Molecular characterization of *Bdellovibrio* spp. based on PCR amplification of 16S rDNA.

The amplification of the 16S rRNA gene fragment for the detection of *Bdellovibrio* spp. was successful for all the isolated *Bdellovibrio* strains using primer (BbsF216 and BbsR707) that amplified 492 bp fragment of the 16S rRNA gene with reference strain *B. bacteriovorus* HD100 serving as positive control (Figure 3. 11). The BLAST search analysis of the 16S rRNA gene sequences in the NCBI and RDP database showed that the *Bdellovibrio* strains belong to the class deltaproteobacteria with four strains including SKB1291214, SSB218315, SKUVM1 and SCRB3 belonging to the genus *Bdellovibrio*. The BLAST search analysis showed that strain SSFD2 belong to the family Bacteriovoraceae and genus *Bacteriovorax*. The result for the PCR amplification of the 492 bp fragment of the 16S rRNA gene for the four *Bdellovibrio* spp. and one *Bacteriovorax* sp. is shown in Figure 3. 12. For *Bdellovibrio* spp. SKB1291214 and SSB218315, consensus sequences were generated from successful PCR amplified (Figure 3. 13), cloned and sequenced 16S rRNA gene fragments of 1493 bp in size. Meanwhile for *Bdellovibrio* sp. SKUVM1, SCRB3, and *Bacteriovorax* sp. SSFD2, consensus sequences were generated from directly sequenced 1493 bp fragment of the 16S rRNA gene. The consensus sequences of the 16S rRNA gene is shown in Figure 3.14 and 3. 15.

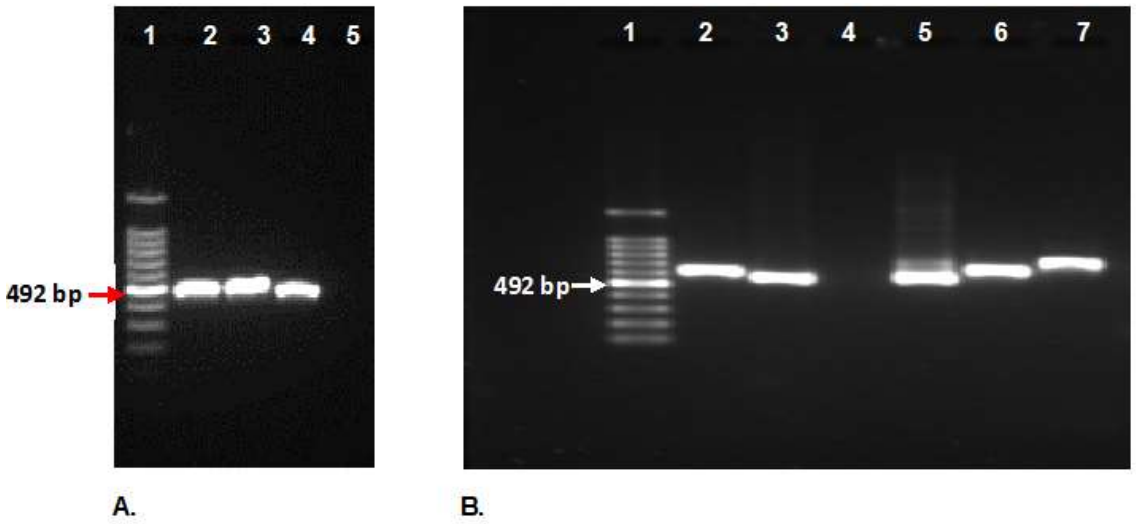


Figure 3. 11 (A). Agarose gel image of the PCR amplification for the detection of *Bdellovibrio* spp. SKB1291214 and SSB218315 using primer that amplified 492 bp of the 16S rRNA gene fragment with reference strain *B. bacteriovorus* HD100 serving as positive control. [1.] 100 bp marker, [2.] *B. bacteriovorus* HD100, [3.] *Bdellovibrio* sp. SKB1291214, [4.] *B. bacteriovorus* SSB218315, [5.] Sterile milliQ water. (B). Agarose gel image of the PCR amplification for the detection of the four *Bdellovibrio* and one *Bacteriovorax* spp. using primer that amplified 492 bp of the 16S rRNA gene fragment [1.] 100 bp marker, [2.] *Bdellovibrio* sp. SKB1291214, [3.] *B. bacteriovorus* SSB218315, [4.] sterile milliQ wáter, [5.] *Bdellovibrio* sp. SKUVM1, [6.] *Bdellovibrio* sp. SCRB3, [7.] *Bacteriovorax* sp. SSFD2.

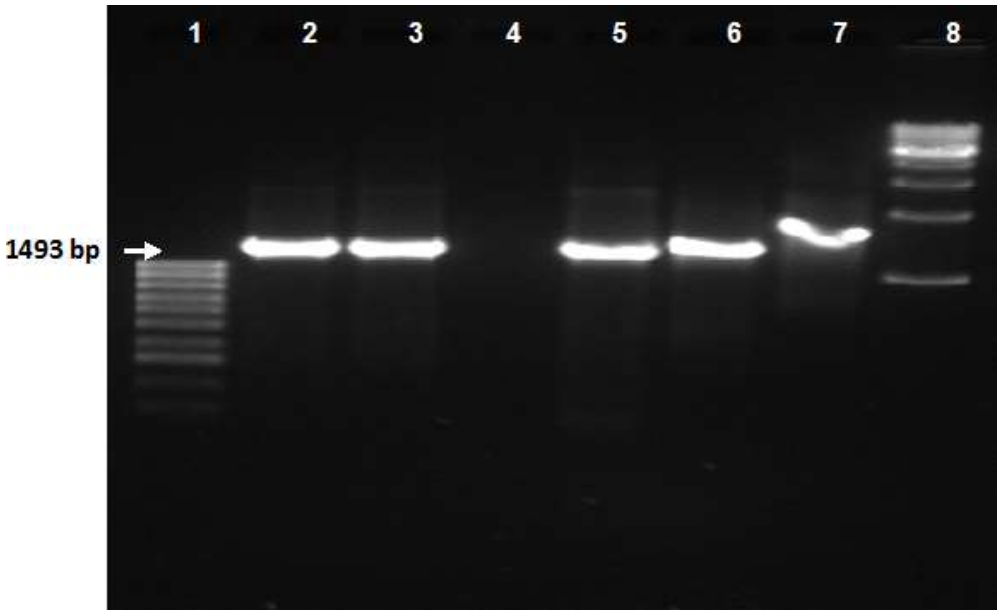


Figure 3. 12. Agarose gel image of the PCR amplification of 1493 bp fragment of the 16S rRNA gene for the *Bdellovibrio* spp. and *Bacteriovorax* sp. [1.] 100 bp marker, [2.] *Bdellovibrio* sp. SKB1291214, [3.] *B. bacteriovorus* SSB218315, [4.] sterile milliQ wáter, [5.] *Bdellovibrio* sp. SKUVM1, [6.] *Bdellovibrio* sp. SCRB3, [7.] *Bacteriovorax* sp. SSFD2, [8.] 1 kb marker.

>16S rRNA gene sequence for *Bdellovibrio* sp. SKB291214 (1460 bp) generated using 16S rRNA primer that amplified 1493 bp of the 16S rRNA fragment

ACAAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGGGGAAAGCTTTCGGGTGAGTACTAGTGGCGC
ACGGGTGAGTAACGCGTGGATAATCTGCCTTAGAGTGGGGGATAACGAATCGAAAGATTCGCTAATACCGC
ATAAGACCACAAGAACTGCGGTTCAAGGGGTCAAAGGTTTTTCGCTCTAAGATGAGTCCGCGTAAGATTAG
CTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATCTTTAACTGGTCTGAGAGGATGATCAGTCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCACAATGGAGGAAACTCTGA
TGCAGCGACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCGCAGGGGAATAACACAATGA
ATGTACCCTGTAAGAAAGGATCGGCTAACTTCGTGCCAGCAGCCGCGGTAAGACGAGGGATCCTAGCGTTG
TTCGGAATTATTGGGCGTAAAGCGGGTGTAGGTGGCTATGTAAGTCAGGTGTGAAAGCCTGGGGCTCAACC
CCAGAAGTGCATCTGATACTGCGTAGCTTGAGTGCTAGAGAGGATAGTAGAATTCTTGGTGTAGTGGTAAA
ATACGTAGATATCAAGAGGAATACCGGTGGCGAAGGCGGCTATCTGGCTAGACACTGACACTCAGACCCGA
AAGTGCGGGGATCAAACAGGATTAGATACCCTGGTAGTCCGCACCATAAACGATGGATACTTGTTGTTGGA
GGTATTGACCCCTTCAGTGACGAAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCAAAGAAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTAGGCTTGACATGTACTGGAATATTGGCGGAAACGCCGTAGCCCGCAAGGGTCGGTACACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTACAT
TTAGTTGCCAGCATTCAGTTGGGCACTCTAGATGGACTGCCGGTGTTAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGCCTAGGGCTACACACGTGCTACAATGGTAGTCACAAACTGAAGCGAAG
TCGTGAGATGGAGCAAATCGGATAAAAGCTATCTAAGTTCAGATTGGTCTCTGCAACTCGAGACCATGAAG
TTGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCATGAAAGTTGGTTGTACCAGAAGTCGCTGCGCTAACCGCAAGGAGGCAGGCGCCCAAGGTATGG
TCGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGG

>16S rRNA gene sequence for *B. bacteriovorus* SSB18315 (1459 bp) generated using 16S rRNA primer that amplified 1493 bp of the 16S rRNA fragment

ACAAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGGGGAAAGCTTTCGGGTGAGTACTAGTGGCGC
ACGGGTGAGGAACGCGTGGATAATCTGCCTTAGAGTGGGGGATAACTAGTCGAAAGATTAGCTAATACCGC
ATAAGACCACAGGAGCTGCGGCTCAAGGGGTCAAAGGTTTTTCGCTCTAAGATGAGTCCGCGTAAGATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCTTTAACTGGTCTGAGAGGATGATCAGTCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCACAATGGAGGAAACTCTGA
TGCAGCGACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCGCAGGGGAATAACACAATGA
ATGTACCCTGTAAGAAAGGATCGGCTAACTTTGTGCCAGCAGCCGCGGTAAGACGAGGGATCCTAGCGTTG
TTCGGAATTATTGGCGTAAAGCGGATGTAGGTGGCTTTGTAAGTCAGATGTGAAAGCCCAGGGCTCAACCC
TGGAAGTGCATTTGATACTGCGAAGCTTGAGTGTCGGAGAGGTTACTAGAATTGTTGGTGTAGTGGTGAAA
TACGTAGATATCAACAGGAATACCGGAGGCGAAGGCGGGTAACTGGCCGAACACTGACACTGAGATCCGAA
AGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTTGTTGTTGGAG
GTATTGACCCCTTCAGTGACGAAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAA
ACTCAAAGAAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC
CTTACCTAGGCTTGACATGTACTGGAAGATTGGCAGAAATGTCGTCGCCCGCAAGGGTCGGTACACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGCATT
TAGTTGCCAGCATTCAGTTGGGCACTCTAGATGGACTGCCGGTGTTAAACCGGAGGAAGGTGGGGATGACG
TCARGTCCTCATGGCCCTTATGCCTAGGGCTACACACGTGCTACAATGGTAGTCACAGAGCGAAGCTAAGC
CGCGAGGTAGAGCAAATCGCTTAAAAGCTATCTAAGTTCAGATTGATCTCTGCAACTCGAGATCATGAAGT
TGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCATGAAAGTCGGCTGTACCAGAAGTCGCTGCGCTAACCGTAAGGAGGCAGGCGCCCAAGGTATGGT
CGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGG

Figure 3. 13. The generated 16S rRNA consensus sequences for the isolated *Bdellovibrio* spp. SKB1291214 and SSB218315

>16S rRNA gene sequence for *Bdellovibrio sp.* SKUVM1 (1107 bp) generated using 16S rRNA primer that amplified 1493 bp of the 16S rRNA fragment

TATATGCAGTCGACGGGGTAGCAATACCTAGTGGCGCACGGGTGAGTAACGCGTGGATAATCTGCCTTAGA
GTGGGGGATAACTAGTCGAAAGATTAGCTAATACCGCATAAGACCACAAGAACTGCGGTTCAAGGGGTCAA
AGGTTTTTCGCTCTAAGATGAGTCCGCGTAAGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACG
ATCTTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTAGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCGACGCCGCGTGAGTGATGAAGGCCTTCG
GGTCGTAAAGCTCTGTCGCAGGGGAATAACACAATGAATGTACCCTGTAAGAAAGGATCGGCTAACTTCGT
GCCAGCAGCCGCGGTAAGACGAGGGATCCTAGCGTTGTTCGGAATTATTGGGCGTAAAGCGGATGTAGGTG
GCTTTGTAAGTCAGATGTGAAAGCCCAGGGCTCAACCCTGGAAGTGCATTTGATACTGCGAAGCTTGAGTG
TCGGAGAGGTTACTAGAATTGTTGGTGTAGTGGTGAAATACGTAGATATCAACAGGAATACCGGAGGCGAA
GGCGGGTAACTGGCCGAACACTGACACTGAGATCCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGGATACTTGTTGTTAGAGGTATTGACCCCTTTAGTGACGAAGCTAACGCGT
TAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGAAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAGGCTTGACATGTACTGGAAGATTGG
CAGAAATGTCGTCGCCGTAAGGGTCGGTACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCAGATTTGG
GTAAGTCCGAACGAGACCCCTGCATTAGTTGCTTTGGCAATGGATGCGGTAACCGGAAGGGACAGTGGCCT
TAGGCTGGAAACCAACAATCTTAACCCCCACTCATTTCTCTT

>16S rRNA gene sequence for *Bdellovibrio sp.* SCRB3 (1092 bp) generated using 16S rRNA primer that amplified 1493 bp of the 16S rRNA fragment

CGCCACTGCAAGTCGAACGGGGAAAGCTTTCGGGTGAGTACTAGTGGCGCACGGGTGAGGAACGCGTGGAT
AATCTGCCTTAGAGTGGGGGATAACTAGTCGAAAGATTAGCTAATACCGCATAAGACCACAGGAGCTGCGG
CTCAAGGGGTCAAAGGTTTTTCGCTCTAAGATGAGTCCGCGTAAGATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCGACGATCTTTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTAGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCGACGCCGCGTGAGTG
ATGAAGGCCTTCGGGTCGTAAAGCTCTGTCGCAGGGGAATAACACAATGAATGTACCCTGTAAGAAAGGAT
CGGCTAACTTCGTGCCAGCAGCCGCGGTAAGACGAGGGATCCTAGCGTTGTTCGGAATTATTGGGCGTAAA
GCGGATGTAGGTGGCTTTGTAAGTCAGATGTGAAAGCCCAGGGCTCAACCCTGGAAGTGCATTTGATACTG
CGAAGCTTGAGTGTCGGAGAGGTTACTAGAATTGTTGGTGTAGTGGTGAAATACGTAGATATCAACAGGAA
TACCGGAGGCGAAGGCGGGTAACTGGCCGAACACTGACACTGAGATCCGAAAGCGTGGGGATCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTTGTTGTTGGAGGTATTGACCCCTTCAGTGAC
GAAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGAAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAGGCTTGACATGT
ACTGGAAGATTGGCAGAAATGTCGTCGCCGCAAGGGTCGGTACACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGGAGATGTTGGTAAGCCCCAGACCCCTGTAGTTGCTCTGGATGGATGGGAAGGGATCTTGGCCTTA
GGGACCCCCAAATCGCTTTGGGGCTCT

Figure 3. 14. The generated 16S rRNA consensus sequences for the isolated *Bdellovibrio* spp. SKUVM1 and SCRB3.

>16S rRNA gene sequence for *Bacteriovorax* sp. SSFD2 (1443 bp) generated using 16S rRNA primer that amplified 1493 bp of the 16S rRNA fragment

CCGTCATCATTCCGACCGTAGACGCTCCCCTCCTTGCGGTTAGGGCCACGGCTTCAGGTAAGAACAACTCC
CATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTA
GCGATTCCAACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATACACTTTTTGAGATTTGCTC
CCCCTCACAGGTTCGCTTCCCTTTGTATGTACCATTGTATTACGTGTGTAGCCCTGGACATAAGGGCCATG
AGGACTTGACGTCATCCCCACCTTCCTCCTGGTTAACCCAGGCAGTCTCCCTAGAGTGCCCAACTTAATGC
TGGCAACTAAGGATAGGGGTTGCGCTCGTTGCGAGACTTAACCCAACATCTCACGACACGAGCTGACGACA
GCCATGCAGCGCCTCTCTCTACATTCCCCGAAGGGCACTCCATCTTTTGGGACGGATTCGTAGGAGTTCAA
GCCCAGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATAATCCACCGCTTGTGCGGGCCCCCGTCAA
TTCCTTTGAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGAGCACTTAATGCGTTTGCGTCGACACGGAA
AAGGTCAAGTTCCCCATATCTAGTGCTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCGCCTCAGCGTCAATACTCATCCAGGAAGGCGCCTTCGCCTCTGGTGTTCCTTCGCA
TCTCTACGGATTTTACCCCTACATGCGAAATTCCCCTTCCCCCTCTGAGATTCTAGATAAGCAGTTTCAGA
CGCAGTTCCAGGGTTGAGCCCTGGGATTTCACATCTGACTTGCATATCCGCCTGCGCGCGCTTTACGCCCA
GTAAATCCGAATAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTCCTT
TTATGGTACCATCAAATAATCGGCCTATTAGACCAACTACCATTTTTCCCATATGACAGAGCTTTACAACC
CGAAGGCCTTCCTCACTCACGCGGCGTTGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCCACTGCT
GCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATTCTCTCAAACCAGTTATACAT
CGTTGCCTTGGTAGGCCGTTACCCCACCAACAAGCTAATGTACCGCAGGCCCATCCATCGATGAATGCTTA
CAAGTAGAGGCATTCTTTCTACTGCTACTTTCAAATTTGCAGTACGTATGCGGTATTAGCTCGAATTTCTT
CGAGTTATCCCCCATCGAAGGGCAGGTCACCTACGTGTTACTCACCCGTGCGCCACTTTACTCATCCCGAA
GGACTTTCGCGTTCGACTGCAGG

Figure 3. 15. The generated 16S rRNA consensus sequences for the isolated *Bacteriovorax* sp.

3.6.2. Nucleotide sequence accession numbers

The 16S rRNA gene sequence data obtained in this study was processed with online software Decipher® (Wright *et al*. 2012) to check chimeras and have been submitted to GenBank Databases under accession numbers KT852580.1 and KT807464.1 for *Bdellovibrio* strain SSB218315 and *Bdellovibrio* strain SKB1291214, respectively. Details of data submission can be found at GenBank: www.ncbi/nlm.nih.gov.

3.6.3. Molecular characterization of *Bdellovibrio* spp. based on PCR amplification of host interaction (*hit*) locus.

The *hit* locus was successfully amplified only in *Bdellovibrio* spp. SSB218315 and SCRB3 (Figure 3. 16). The BLAST analysis showed that the consensus sequence has 93 % identity *with B. bacteriovorus hit* locus with a significant e-value of 0.0. The consensus sequences of *hit* locus is shown in Figure 3. 17.

The molecular characteristics of the *Bdellovibrio* spp. isolated in Mexico, Tamaulipas, Mexico based on PCR amplification of 16S rDNA and hit locus is summarized in Table 3. 4.

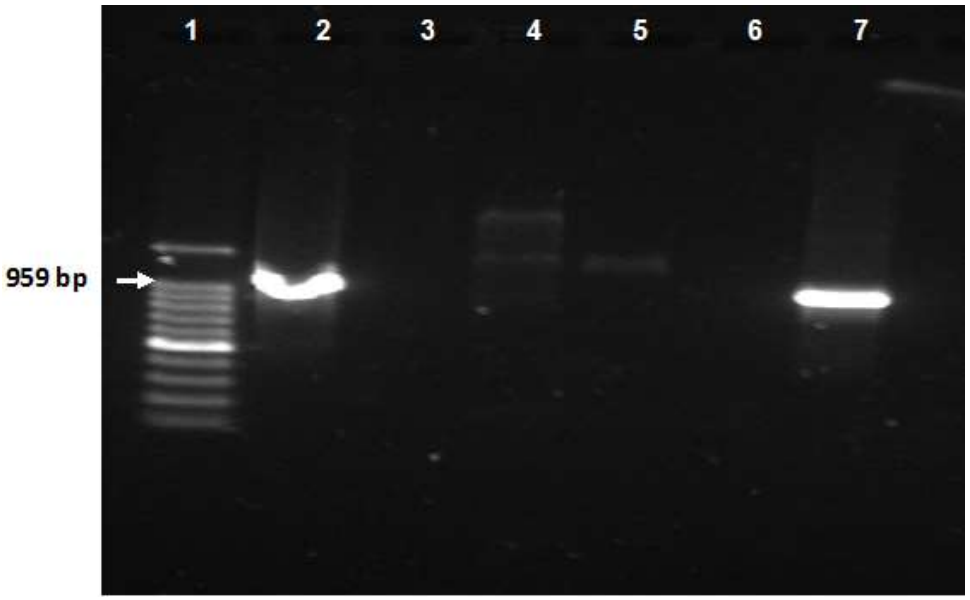


Figure 3. 16. Agarose gel image of the PCR amplification of 959 bp fragment of the *hit* locus gene for the *Bdellovibrio* spp. and *Bacteriovorax* sp.[1.] 100 bp marker, [2.] *B. bacteriovorus* SSB218315, [3.] *Bdellovibrio* sp. SKB1291214, [4.] *Bdellovibrio* sp. SKUVM1, [5.] *Bacteriovorax* sp. SSFD2, [5.] sterile milliQ wáter, [7.] *Bdellovibrio* sp. SCRB3

>Host interaction (*hit*) locus sequence of *B. bacteriovorus* SSB218315 (954 bp) generated using *hit* primer that amplified 959 bp of the *hit* locus fragment

TTAATTTGGGACATACAGCGCGCGGACGGTCAATATTACCTGGCGTGTGATGACGACCGTAAACGGCAACG
TCACAGACATCACTTACCATGAGGTCTTTGGCAAACCCGTTTCCATCCGTCGCAATGCCGACCGTATCTCT
TACGAATACTATCCAGATGGCCTGGTGAAGGTGAAAGCCGCACCAAACATGAAGATGGCATTTGAGTACGA
TCCGAAGGTTAAAAAGGTCAGCTCTGTGAACAGCACTTACTTCAATGAAAAAGGTGCCAAGGTGGCCGTTA
AAGCCGCTCAGTTCAAATACGATGGCAAAGGAAACCTTGCCTATGCCCAGAACAGCGATGGCCAGAAGATC
AATATGACATATGATAACCGCGGCCGTATTGCGACCATTACAGATCAGGCCAAGAAAGTGGTTAAAATCGA
GTACGAGGAACGCTATGGGAAGCCATCTGTAGTTACCCGTCCCGGTTTGGGCACAATTGTGGTAAGCTATA
CCAAATGGTGAAATCAGCAAAGTGGACAGCAAAGAGGGGCCATCTGTAGCTATGCAGGTAGCCAGCACATT
TAATAACCTCTTGGATATCATTGCTCCCGCAACTGCGGAACTTTACCTCTAAGGAGAAGGTGATTATATGA
AAAGACTCTTGGTTCTTTCCATCTTGCTGACCCTGGGCTTCTCCTTTGCGGGAACGGCCTCTGCTGACGAA
AATGCCAACCGCCCGGTAAACCCTGGCGAAGACCCGAATGAAGCCTTCAGATCGTCTCCTTTTGAGGCGAC
GACATCGGCTTTGGGTGACTGCCGTGAGTGTATCGCCTACCGTACCGGCGCCACCACGGGCAAAGGCTCCC
GTCGCCATGACGACACTGTTTCCAGAGAAATCAAAGGCTCCAGCGCGACGCCCGGTGGATCTGAGAAATCC
GGCACCGAAGACAGAATCACTTCGTCTAAAA

Figure 3. 17. The generated *hit* locus consensus sequence for *B. bacteriovorus* SSB218315

Table 3. 4. Summary of the information about the *Bdellovibrio* spp. isolated from soil and sewage in Reynosa, Tamaulipas, Mexico including their molecular characteristics based on the amplification of 16S rRNA gene and *hit* locus

SAMPLE	LOCATION	PREY	16SrDNA	*hit* locus
SKB1291214 *Bdellovibrio* sp.	CBG, Reynosa (26.069678N,-98.313108W); Soil	*Klebsiella* sp.	+	-
SSB218315 *B. bacteriovorus*	CBG, Reynosa (26.069446N,-98.312902W); Soil	*Salmonella* sp.	+	+
SCRB3 *Bdellovibrio* sp.	Ranch in Rio Bravo (25.984519N,-98.129108W); Soil	*Citrobacter freundii* CDBB-B-955 (ATCC 8090)	+	+
SKUVM1 *Bdellovibrio* sp.	UVM area, Reynosa (26.066685N,-98.277428W); Sewage	*Klebsiella* sp.	+	-
SSFD2 *Bacteriovorax* sp.	Boulevard Fundadores (26.089304N, -98.292479W); sewage	*Salmonella* sp.	+	-

+ (positive for PCR amplification) ; - (negative for PCR amplification); Rio Bravo is a town that is 23 kilometers away from Reynosa; UVM: Universidad de Valle de Mexico; CBG: Centro de Biotecnología Genómica

3.6.4. Characterization *Bdellovibrio* sp. SKB1291214 and *B. Bacteriovorus* SSB218315 based on Amplified rDNA restriction analysis.

The ARDRA of *Bdellovibrio* spp. SKB1291214 and SSB218315 showed that the two strains have different restriction pattern when the amplified 16S rDNA was cut using *EcoRI* and *HindIII* restriction enzymes (Figure 3. 18). However, same restriction pattern was observed when the 16S rDNA was cut with *BamHI*, *XhoI* and *XbaI* enzymes. *B. bacteriovorus* SSB218315 show same restriction pattern with reference strain *B. bacteriovorus* HD100 for all the restriction enzymes.

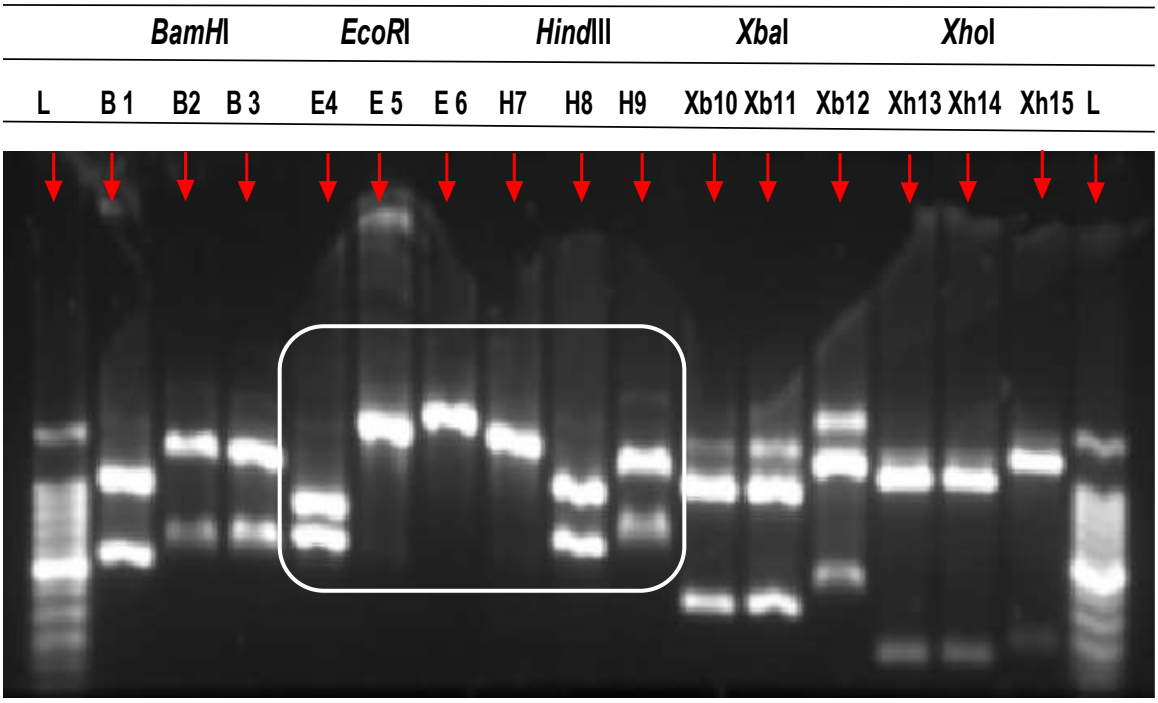


Figure 3. 18. Amplified rDNA restriction analysis (ARDRA) of *Bdellovibrio* sp. SKB1291214, *B. bacteriovorus* SB218315 and HD100 (reference control). L: 100 bp marker

BamHI: [B1]: *Bdellovibrio* sp. SKB1291214, [B2]: *B. bacteriovorus* SSB218315, [B3]: *B.bacteriovorus* HD100; *EcoRI*: [E4]: *Bdellovibrio* sp. SKB1291214, [E5]: *B. bacteriovorus* SSB218315, [E6]: *B.bacteriovorus* HD100.; *HindIII*: [H7]: *Bdellovibrio* sp. SKB1291214, [H8]: *B. bacteriovorus* SSB218315, [H9]: *B.bacteriovorus* HD100.; *XbaI*: [Xb10]: *Bdellovibrio* sp. SKB1291214, [Xb11]: *B. bacteriovorus* SSB218315, [Xb12]: *B.bacteriovorus* HD100.; *XhoI*: [Xh13]: *Bdellovibrio* sp. SKB1291214, [Xh14]: *B. bacteriovorus* SSB218315, [Xh15]: *B.bacteriovorus* HD100.

3.6.5. Phylogenetic analysis and determination of pairwise evolutionary distance or divergence among isolated *Bdellovibrio* spp.

The isolated *Bdellovibrio* strains and *Bacteriovorax* strain clustered with the member of the genus *Bdellovibrio* on the phylogenetic tree. *Bdellovibrio* sp. SKB1291214 shared 99 % identity with uncultured *Bdellovibrio* sp. clone 12L 106 (accession number KP183074.1) (Figure 3. 19). The pairwise evolutionary distance or divergence between these two strains was observed to be 0.01 (Table 3. 5.). It clustered together with two rhizosphere-derived (BRP4 and BEP2) and soil-derived *Bdellovibrio* strains (ETB and SRA9) sharing 97 % identity and a pairwise evolutionary distance of 0.03. It further clustered with the *Bdellocyst* forming *Bdellovibrio* sp. W, however, with 95 % identity and a pairwise evolutionary distance of 0.05. *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 clustered separately on the phylogenetic tree with percentage identity of 96 % and a pairwise distance of 0.05. *B. bacteriovorus* SSB218315 clustered and has 99 % identity with *B. bacteriovorus* strains JSF1, HD100, Tiberius, SRE7 with a pairwise evolutionary distance of 0.00. *Bdellovibrio* sp. SKUVM1 and SCRB3 shared 98 % identity pairwise evolutionary distance of 0.01. However, the two strains (SKUVM1 and SCRB3) shared 94 % (0.05 pairwise divergence) and 98 % (0.01 and 0.00 pairwise divergence for SKUVM and SCRB3 respectively) identity with *Bdellovibrio* sp. SKB1291214 and SSB218315 respectively. The two strains further clustered closer to latter than the former on the phylogenetic tree. The clustering pattern of *Bacteriovorax* sp SSFD2 isolated is atypical. This strain was observed to cluster with the genus *Bdellovibrio* despite belonging to the genus *Bacteriovorax* based on BLAST search analysis of its 16S rRNA gene sequences. However, on the phylogenetic tree, strain SSFD2 clustered more closely to the epibiotic *B. exovorus* JSS, *Bdellovibrio* sp. HEA and JSF1. The pairwise evolutionary distance or divergence between *Bacteriovorax* sp. SSFD2 and other strains considered for the phylogenetic tree construction was observed to range between 0.73 and 1.03.

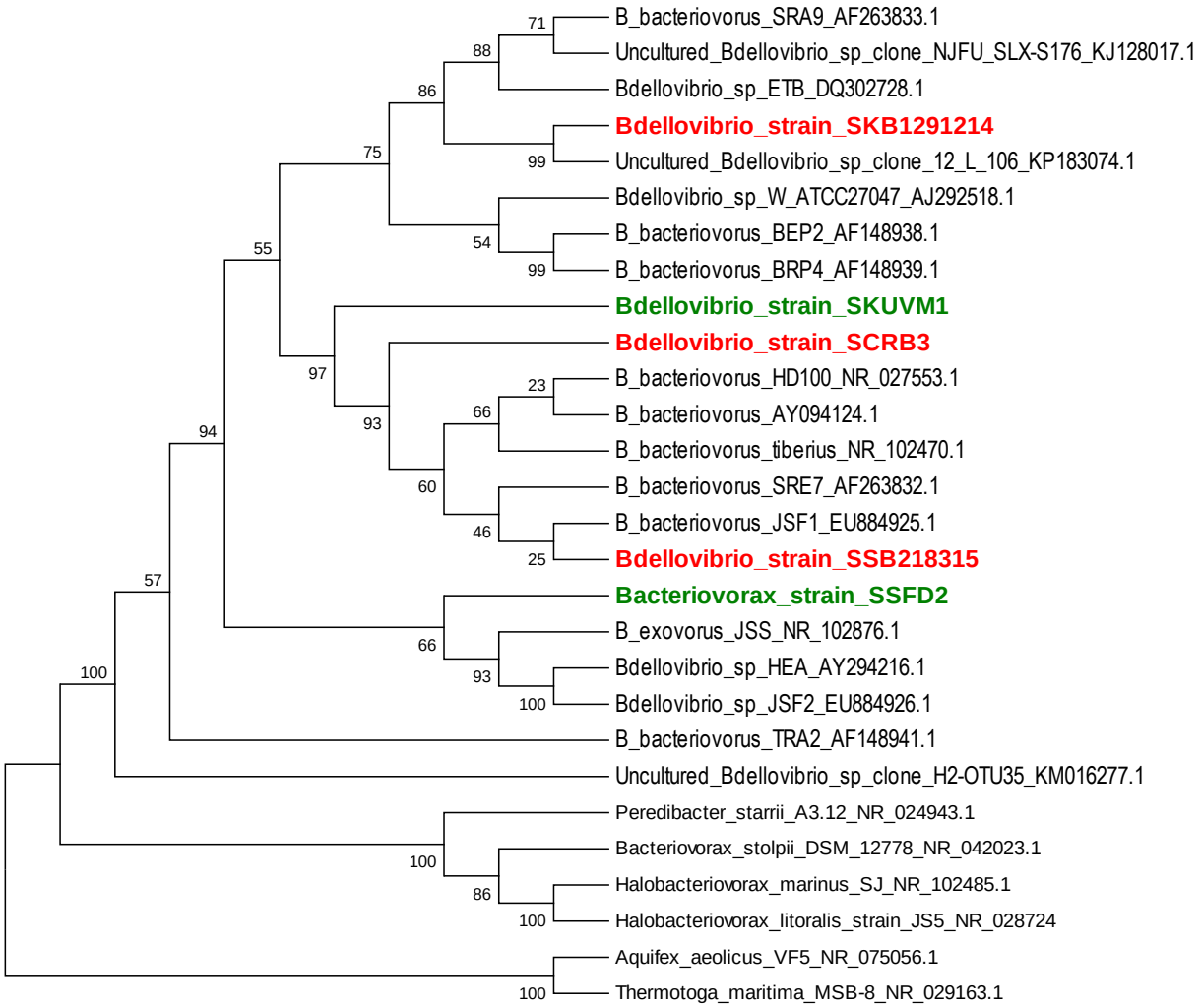


Figure 3. 19. The evolutionary history of BALOs inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 28 16S rRNA gene nucleotide sequences. Evolutionary analyses were conducted in MEGA6. *Thermotoga maritima* 16S rRNA sequence was used as an external group.

Bdellovibrio_strain_SKB1291214																												
Bdellovibrio_sp_W_ATCC27047_AJ292518.1	0.05																											
B_bacteriovorus_tiberius_NR_102470.1	0.05	0.05																										
B_bacteriovorus_HD100_NR_027553.1	0.05	0.05	0.00																									
B_bacteriovorus_JSF1_EU884925.1	0.05	0.05	0.00	0.00																								
B_bacteriovorus_SRA9_AF263833.1	0.03	0.05	0.04	0.04	0.04																							
B_bacteriovorus_SRE7_AF263832.1	0.05	0.05	0.00	0.00	0.00	0.04																						
B_bacteriovorus_TRA2_AF148941.1	0.08	0.08	0.05	0.05	0.05	0.07	0.05																					
B_exovorus_JSS_NR_102876.1	0.10	0.09	0.08	0.08	0.08	0.10	0.08	0.09																				
Bdellovibrio_sp_ETB_DQ302728.1	0.03	0.05	0.04	0.04	0.04	0.01	0.04	0.07	0.10																			
Bdellovibrio_sp_HEA_AY294216.1	0.09	0.09	0.06	0.06	0.06	0.09	0.06	0.08	0.04	0.09																		
Bdellovibrio_sp_JSF2_EU884926.1	0.10	0.09	0.07	0.07	0.07	0.09	0.07	0.09	0.04	0.09	0.01																	
Bacteriovorax_stolpii_DSM_12778_NR_042023.1	0.23	0.22	0.22	0.22	0.22	0.24	0.22	0.22	0.23	0.25	0.22	0.22																
Peredibacter_starrii_A3.12_NR_024943.1	0.22	0.22	0.21	0.21	0.21	0.23	0.21	0.21	0.23	0.23	0.21	0.21	0.13															
Halobacteriovorax_marinus_SJ_NR_102485.1	0.22	0.22	0.22	0.22	0.22	0.23	0.22	0.21	0.22	0.24	0.22	0.22	0.11	0.14														
Aquifex_aeolicus_VF5_NR_075056.1	0.34	0.33	0.33	0.33	0.33	0.34	0.33	0.30	0.33	0.34	0.34	0.33	0.34	0.35	0.33													
Thermotoga_maritima_MSB-8_NR_029163.1	0.29	0.28	0.27	0.27	0.27	0.28	0.27	0.26	0.27	0.29	0.28	0.28	0.30	0.32	0.30	0.22												
Bdellovibrio_strain_SSB218315	0.05	0.05	0.00	0.00	0.00	0.04	0.00	0.06	0.08	0.04	0.07	0.07	0.23	0.21	0.22	0.33	0.27											
Uncultured_Bdellovibrio_sp_clone_NJFU_SLX-S176_KJ128017.1	0.03	0.05	0.04	0.04	0.04	0.01	0.04	0.07	0.10	0.01	0.09	0.09	0.24	0.23	0.24	0.34	0.29	0.04										
Uncultured_Bdellovibrio_sp_clone_H2-OTU35_KM016277.1	0.09	0.09	0.07	0.07	0.07	0.08	0.07	0.04	0.10	0.09	0.08	0.08	0.21	0.18	0.20	0.30	0.26	0.07	0.09									
Uncultured_Bdellovibrio_sp_clone_12_L_106_KP183074.1	0.01	0.05	0.05	0.05	0.05	0.03	0.05	0.08	0.11	0.03	0.10	0.10	0.24	0.23	0.23	0.34	0.30	0.05	0.03	0.09								
B_bacteriovorus_AY094124.1	0.05	0.05	0.00	0.00	0.00	0.04	0.00	0.05	0.08	0.04	0.06	0.07	0.22	0.21	0.22	0.33	0.27	0.00	0.04	0.07	0.05							
Halobacteriovorax_litoralis_strain_JS5_NR_028724	0.22	0.22	0.21	0.21	0.21	0.23	0.21	0.20	0.20	0.23	0.20	0.20	0.11	0.15	0.08	0.34	0.31	0.21	0.23	0.20	0.23	0.21						
Bdellovibrio_strain_SKUVM1	0.05	0.05	0.01	0.01	0.01	0.04	0.01	0.07	0.08	0.05	0.07	0.08	0.23	0.22	0.22	0.34	0.28	0.01	0.05	0.08	0.06	0.01	0.22					
Bdellovibrio_strain_SCRB3	0.05	0.05	0.00	0.00	0.00	0.04	0.00	0.06	0.08	0.04	0.07	0.07	0.23	0.22	0.22	0.33	0.28	0.00	0.04	0.07	0.06	0.00	0.22	0.01				
Bacteriovorax_strain_SSFD2	0.74	0.74	0.73	0.73	0.73	0.74	0.73	0.76	0.73	0.75	0.74	0.74	0.95	0.92	0.89	1.03	0.99	0.73	0.75	0.76	0.74	0.73	0.96	0.74	0.73			
AF148938.1_B_bacteriovorus_BEP2	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.05	0.09	0.04	0.07	0.08	0.23	0.22	0.22	0.32	0.27	0.03	0.04	0.07	0.04	0.03	0.22	0.03	0.03	0.73		
AF148939.1_B_bacteriovorus_BRP4	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.05	0.09	0.04	0.07	0.08	0.23	0.22	0.22	0.32	0.27	0.03	0.04	0.07	0.04	0.03	0.22	0.03	0.03	0.73	0.00	

Table 3. 5. Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 28 16S rRNA gene nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

DISCUSSION

Bdellovibrio spp. were successfully isolated from soil and sewage samples using double layer agar plating technique and dilute nutrient broth agar (DNB) as the medium. Plaque development was observed within 2-7 days on DNB with three bacteria of the member Enterobacteriaceae (*Klebsiella* sp., *Salmonella* sp. and *Citrobacter freundii*) serving as prey. The characteristics of the soil samples suggested their good agricultural value while the sewage samples were polluted with organic matter. A total of five BALOs were isolated including four strains of genus *Bdellovibrio* and one strain that belong to genus *Bacteriovorax.* Several attempts to isolate *Bdellovibrio* spp. from the guts and dung of ruminant animals using direct and enrichment method were unsuccessful. *Bdellovibrio* spp. have been reported to be frequently encountered in soil representing about 80 % of all BALOs in soil community (Fulthorpe *et al*., 2008). Sewage has also been described as a source to isolate *Bdellovibrio* spp. (Koval & Hynes, 1991; Williams *et al*., 1995). However, the ecological role of *Bdellovibrio* spp. in the different niche where they are encountered is not well understood, perhaps serving as an "ecological balancer" as described by Lebba *et al*. (2014).

Different media have been described for the isolation of *Bdellovibrio* spp. (Stolp and Starr, 1963, Lambert and Sockett, 2008; Jurkevitch, 2012). This study further supports the use of DNB agar in double agar plating technique as an effective method of isolating *Bdellovibrio* strains from the soil. The DNB agar (0.08 % nutrient broth amended with 3 mM MgCl2. $6H_2O$, 2 mM $CaCl_2$. $2H_2O$, 0.6 % agar for top agar and 1.9 % for bottom agar [pH 7.2 – 7.4]) contained little amount of nutrient so as not to support the rapid growth of the prey. The rapid growth rate of the prey cells gives bacteriophage ample chance to develop thereby interfering with slower growing *Bdellovibrio* spp. Furthermore, less amount of nutrient in the medium also limit the growth rate of bacterial contaminants. The addition of $MgCl_2$ and $CaCl_2$ in the DNB medium increase the competence of prey cells to *Bdellovibrio* spp. predation. Prey choice is also one of the crucial factors in the successful isolation of *Bdellovibrio* spp. It was observed in this study that chances of *Bdellovibrio*

spp. preying upon members of the Enterobacteriaceae is very high (SKB1291214: 13/18 = 72 %; SSB218315: 16/18 = 89 %; SKUVM1 and SCRB3: 18/18 = 100 %) suggesting that they can be excellent prey for routine and successful isolation of *Bdellovibrio* spp. from environmental samples. Also, *Bdellovibrio* sp. SKB1291214 that preyed upon the lowest number of bacteria considered for prey range analysis in this study had its prey range limited to the members of the group Enterobacteriaceae. This further showed that Enterobacteriaceae are frequently susceptible to *Bdellovibrio* spp. predation. Furthermore, Gram-negative bacteria that differ from *Bdellovibrio* strains most especially in size and motility, such as *Klebsiella* spp. could be considered suitable for the successful isolation of *Bdellovibrio* strains. This will allow easy differentiation of *Bdellovibrio* strains from their prey under the microscope.

Plaque morphology (including days required for plaque development - most times more than 24 h), light microscopy to view highly motile *Bdellovibrio* spp., and the use of powerful microscope such as atomic force microscope or electron microscope are the ways to phenotypically identify prey dependent *Bdellovibrio* spp. Since *Bdellovibrio* spp. are cultured in a two-member culture system, the presence of prey cells that may accidentally pass through filters during filtration process may interfere with biochemical test results carried out on prey dependent *Bdellovibrio* spp.

Salmonella infections have been described as one of the major public health concern worldwide. It accounts for 93.8 million foodborne illnesses and 155,000 deaths per year (Eng *et al*., 2015). It has been linked to infections such as Gastroenteritis, bacteraemia and enteric fever (Majowicz *et al*. 2010). *Klebsiella* spp. are opportunistic pathogens that are implicated in infections such as sepsis, pneumonia, urinary tract infections, and hepatic abscess (Hennequin *et al*., 2007). *Citrobacter freundii* are usually commensals in the guts of humans and animals (Bai *et al*., 2012). However, some isolates have been reported to harbor virulent factors that make them cause diarrhea in humans (Al-Hissnawy *et al*., 2014). Furthermore, *Salmonella* spp., *Klebsiella* spp., and *Citrobacter* spp., have been reported to have the ability to become multidrug resistant (Eng *et al*., 2015; Moradigaravand *et al*., 2017; Majewski *et al*., 2017; Negus *et al*., 2017). The emergence of multidrug resistant bacteria and dearth of novel antibiotics to treat bacterial infections is an

indication for the need to search alternative to antibiotics in the treatment of bacterial infections. The ability of *Bdellovibrio* spp. isolated in the study to lyse *Klebsiella* sp., *Salmonella* sp. and *Citrobacter freundii* suggested the possibility of using these *Bdellovibrio* strains to control pathogenic and multidrug resistant strains of these study preys.

Most of the prey considered in this study belong to the phylum γ-proteobacteria with two α-proteobacteria (*Rhizobium leguminosarum* and *Agrobacterium tumefaciens*) and only one β-proteobacteria (*Alcaligenes* sp). *Bdellovibrio* spp. are not prey specific with prey range varying with strains (Schelling and Conti, 1978). The prey range of the *Bdellovibrio* spp. does not follow a specific pattern except for *Bdellovibrio* sp. SCRB3 and SKUVM1 that exhibited same prey range pattern. Preferential predation has been reported in BALOs (Li *et al*. 2011) and moreover, *Bdellovibrio* spp. have the ability to reversibly attach to and detach from susceptible and non-susceptible prey. But what results into prey penetration is an irreversible attachment to the prey cell. However, the mechanism involved in the selection of prey in *Bdellovibrio* spp. is not well understood.

The *Bdellovibrio* spp. exhibited some preference for the bacterial isolates belonging to the family Enterobacteriaceae and coupled with the fact that *Bdellovibrio* strains have been isolated from guts of mammals (Schwudke *et al*. 2001; Lebba *et al*. 2013), they could be used to stabilize intestinal bacterial flora perhaps as probiotics. Furthermore, *Rhizobium leguminosarum* lives symbiotically with root of leguminous plants helping in nitrogen fixation which in turn aid plant growth (Argaw and Mnalku, 2017). Though *Bdellovibrio* spp. that has the ability to prey upon *Rhizobium* spp. have been reported (Parker *et al*., 1970) but the inability of the study *Bdellovibrio* spp. to prey upon *Rhizobium leguminosarum* suggests the possibility of using the two bacteria synergistically to help plant growth. None of the *Bdellovibrio* strains formed plaque on the six gram-positive bacteria viz. genera *Staphylococcus* and *Bacillus* considered in this study except for *Bdellovibrio* spp. SKUVM1 and SCRB3 which caused a reduction in turbidity when cocultured with *Staphylococcus epidermidis* in a microtiter plate. One of the setbacks that could limit the application of *Bdellovibrio* strains is their inability to attack or utilize gram-positive bacteria as prey as equally

observed in this study with the inability of the study strains to form plaque on all the gram-positive bacteria considered for the prey range analysis. However, the ability of *Bdellovibrio bacteriovorus* HD100 to survive in the presence of *Staphylococcus aureus* using epibiotic mode of attack has been reported (Lebba *et al*., 2014).

The BLAST search analysis of the 16S rRNA gene sequences in the NCBI and RDP database showed that the *Bdellovibrio* strains belong to the class deltaproteobacteria with four strains including SKB1291214, SSB218315, SKUVM1 and SCRB3 belonging to the genus *Bdellovibrio*. The BLAST search analysis showed that strain SSFD2 belong to the family Bacteriovoraceae and genus *Bacteriovorax*. The isolated *Bdellovibrio* spp. clustered together with the other members of the genus *Bdellovibrio* on the phylogenetic tree. However, genetic diversity was observed among the *Bdellovibrio* spp. isolated in this study. *Bdellovibrio* sp. SKB1291214 clustered with uncultured *Bdellovibrio* sp. clone 12L 106 (99 %), and clustered separately from other isolated *Bdellovibrio* spp. with genetic distance of 0.05. The genetic diversity observed among the isolated *Bdellovibrio* spp. further supported the report that the genus *Bdellovibrio* is heterogeneous with members exhibiting phylogenetic diversity (Jurkevitch *et al*., 2000). The heterogeneity that exists among members of the genus *Bdellovibrio* was also observed with the result of ARDRA done on *Bdellovibrio* spp. SKB1291214 and SSB218315.

In addition, the distant relationship that has been reported to exist between the marine; family Bacteriovoraceae and terrestrial; family Bdellovibrionaceae (including the freshwater) groups of "*Bdellovibrio* and like organisms" (BALOs) as reported by Baer *et al*. (2000) and Jurkevitch *et al*. (2000) can also be inferred from the phylogenetic tree. These two groups of BALOs were initially grouped together as Bdellovibrionaceae but later separated into two groups based on differences in the characteristics including variations in G+C content, prey preference, and response to salinity.

The *hit* locus was successfully amplified in *Bdellovibrio* spp. SSB218315 and SCRB3 strains but not in *Bdellovibrio* spp. SKB1291214, SKUVM1 strains, and *Bacteriovorax* sp. SSFD2. Sequencing and analysis of the amplified product from *Bdellovibrio* strain SSB218315 further confirmed it to be *hit* locus. The blast analysis showed the study *Bdellovibrio* strains exhibiting 97%

identity with two rhizosphere-derived *B. bacteriovorus* strains BEP2 and BRP4 reported by Jurkevitch *et al*. (2000). However, these two strains clustered more closely to *Bdellovibrio* strain SKB1291214 than the other *Bdellovibrio* spp. on the phylogenetic tree. And interestingly, *hit* locus was not successfully amplified in this two reported strains (*B. bacteriovorus* strains BEP2 and BRP4) when PCR technique was used. This is an indication that *Bdellovibrio* strain SKB1291214 may be a plant rhizosphere-associated strain. Furthermore, since the *hit* locus has been proposed to be restricted to *B. bacteriovorus* (Schwudke *et al*., 2001), electron microscopy may provide more information on *Bdellovibrio* strain SKB1291214. Perhaps, it may be using a different mechanism of action for its predatory activities.

In summary, the *Bdellovibrio* strains isolated from soil and sewage exhibited the ability to prey upon different types of gram-negative bacteria and this attribute could be considered for future use in the control of pathogenic gram-negative bacteria. The differences observed between the *Bdellovibrio* spp. isolated here with respect to amplification of *hit* locus and prey range further support Jurkevitch *et al.* (2000) report that population of *Bdellovibrio* is made up of heterogeneous groups. Therefore, this suggests the need for further characterization and classification of soil-associated *Bdellovibrio* in Mexico for the possibility of grouping them into different subgroups (strains). Finally, with the paucity of information on BALOs research in Mexico, this work is expected to pave way for basic line of research in BALOs with the ultimate goal of utilizing them for biotechnological applications.

CHAPTER FOUR

Objective 2: To carry out phenotypic and molecular characterization of prey-independent (PI) *Bdellovibrio* phenotypes that will be derived from the successfully isolated host-dependent Bdellovibrio strains from objective (1) above.

4.1. INTRODUCTION AND LITERATURE REVIEW

4.1.1. History and description of prey-independent *Bdellovibrio* strains.

Bdellovibrio bacteriovorus was first described as an obligate predatory bacterium (Stolp and Petzold, 1962; Stolp and Starr, 1963). Stolp and his two colleagues made several attempts to isolate PI phenotype that can grow in the absence of prey on nutrient-rich media. Their attempts resulted in the isolation of yellow PI strains that lost motility and predatory activities. However, Shilo and Bruff, (1965) described the PI phenotype of *B.bacteriovorus* strain A3.12 that retained the predatory characteristics. This was achieved by separation of the prey from *B. bacteriovorus* A3.12 via filtration method using 0.45 µm filter, followed by the transfer of prey-free *B. bacteriovorus* A3.12 (~10^9) into nutrient broth or dilute nutrient broth containing heat-killed prey ~10^8 which was then incubated at 30 ^oC with shaking. For the generation of PI phenotype on solid media, ~10^4 of *B. bacteriovorus* A3.12 was cultured on nutrient agar. *B. bacteriovorus* A3.12 and its PI phenotype were reported to be capable of releasing potent protease that could lyse heat-killed bacteria into NB medium. The exoenzyme also had the ability to degrade protease such as coloured collagen preparation Azocoll as well as lyse Gram-positive bacteria such as *Bacillus megaterium*. The presence of phosphate was reported to lead to a rapid loss of the exoenzyme activity as observed in the decomposition of the Azocoll. Similar to the observations of Stolp and his colleagues, Shilo and Bruff further observed a decrease in the predacity of the *B. bacteriovorus* A3.12 PI phenotype after prolonged subcultivation, however with no loss of motility. Aside from that, the technique employed by the duo was not successful for isolating PI *Bdellovibrio* phenotypes from host-dependent *Bdellovibrio* strains such as *B. bacteriovorus* strain 109. Ishiguro (1973) successfully isolated PI phenotype of *B. bacteriovorus* strain 109 and demonstrated the importance of heat-stable growth initiation factor in initiating the growth of PI phenotypes of *B. bacteriovorus* 109. In addition, there are several reports on the influence of prey cell-free extracts in the growth of prey-

dependent (PD) *Bdellovibrio* and development of PI *Bdellovibrio* (Reiner and Shilo, 1969; Horowitz *et al*., 1974; Ishiguro, 1973; Friedberg, 1978).

With the limitations attributed with earlier described techniques for the isolation of PI *Bdellovibrio* phenotypes (development of host colonies that mask small slow-growing *Bdellovibrio* and inability to apply similar techniques to all PD *Bdellovibrio* strains), Seidler and Starr (1969), reported another reliable technique for the isolation of PI *Bdellovibrio* phenotypes. This method involved the propagation of streptomycin-resistant (Sm^r) Bdellovibrios on streptomycin-sensitive (Sm^s) prey cells on peptone-yeast (PYE) agar. The PI *Bdellovibrio* were observed to grow as yellow pleomorphic colonies with heterogeneous sizes ranging from 1-2 mm with an increase in size at room temperature to 2 - 3 mm (Figure 4.1). Furthermore, their biochemical characteristics include the ability to liquefy gelatin, produce ammonia from peptone (PYE broth), and grow at temperature range of 23 to 37 ^oC. They produce catalase, oxidase, proteolytic exoenzymes (as earlier reported), and yellow, wáter-insoluble pigment that was observed to decrease in amount with successive transfer on the prey-free médium. The PI isolates reported by Shilo and Bruff (1969) are not capable of reducing nitrates, or producing índole, and they cannot utilize carbon compounds. Antibiotic susceptibility testing showed that they are sensitive to Altafur, kanamycin (three strains resistant), methenamine mandelate (one strain resistant), neomycin, novobiocin, polymyxin B (two strains resistant), oxytetracycline, and vibriostat 0/129 (one strain resistant). However, they were resistant to colistin (one strain susceptible), sulfisomadine sulfisoxazole (one strain susceptible), isoniazid, sulfadimethoxine mystatin, oleandomycin (one strain susceptible), penicillin (three strains susceptible), sulfadiazine, sulfamethoxy-pyradazine, triple sulfa, and vancomycin. The percentage GC of the strains were reported to be 43 to 51 % (most strains 50 to 51 %). The shape of the PI phenotypes is vibrio (rod) to spiral, and the flagellated cells possess single sheathed polar flagellum.

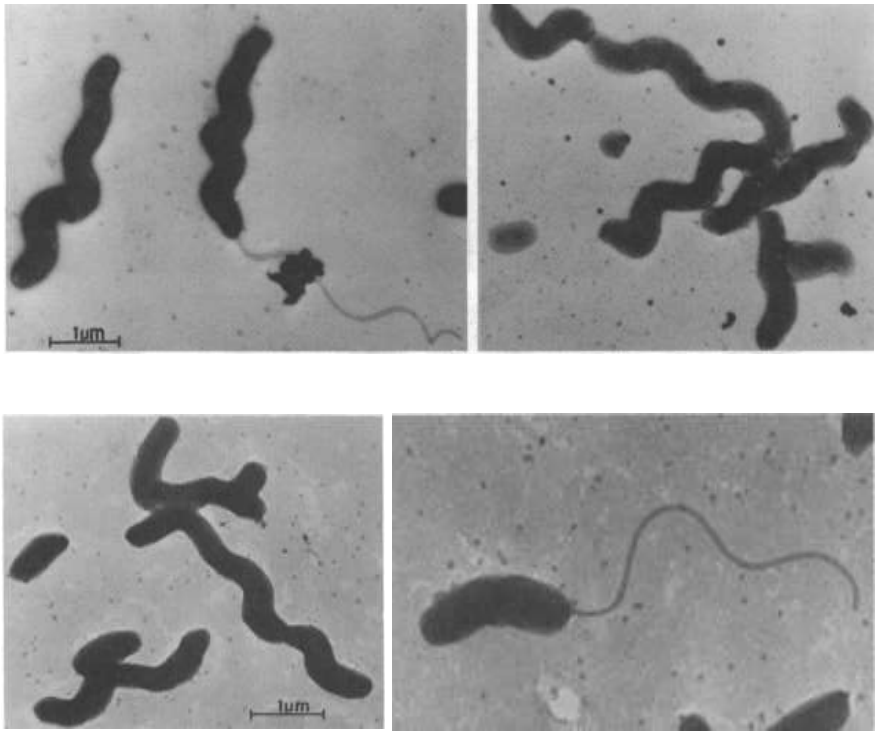


Figure 4. 1. Image showing pleomorphism in prey-independent *Bdellovibrio*. The shapes ranges from vibrio- to spiral-shaped cells. (Seidler and Starr, 1969)

In another experiment, Diedrich *et al*., (1970) isolated PI *B. bacteriovorus* UKi2 from parent PD *B. bacteriovorus* UK by the cultivation of colonies within plaques when double-layer plates were incubated for more than 3 days. This strain (*B. bacteriovorus* UKi2) exhibited similar properties described earlier by Seidler and Starr, (1962) except that the PI colonies of UKi2 were described to be whitish-grey instead of yellow, and can grow saprophytically in prey-free medium with relatively stable predacity. They further observed that the spiral forms of the PI phenotype are non-motile and non-infective. Physiological characteristics of *B. bacteriovorus* UKi2 is shown in Table 4. 1.

Table 4. 1. Some physiological characteristics of *B. bacteriovorus* UKi2 (Diedrich *et al*., 1970)

Test	Result
Catalase	+
Hydrogen sulfide	−
Indole	−
Nitrate reduction	+
Methyl red	−
Voges-Proskauer	−
Acid from 18 carbon sources	−
Hemolysis	+
Gelatin hydrolysis	+
1% Glycine tolerance	−
Sodium chloride tolerance	
3.5%	−
9.0%	−
Relation to oxygen	Microaerophilic
Optimal *p*H range	6.5–8.0
Optimal temperature	25–30 C

Several other techniques have been employed in the isolation of *Bdellovibrio* PI phenotypes. Ferguson *et al.*, (2008) derived PI phenotype by concentrating PD *B. bacteriovorus* 109J on 0.2 µm polycarbonate filter paper placed on LB agar. The PI colonies derived were yellow as earlier observed by other researchers, rod-shaped (with different length) with reduced motility. They also concluded and supported the work of Barel and Jurkevitch, (2001) that mutation is not the only condition needed for the derivation of PI phenotype of *Bdellovibrio*. Dashiff and Kadouri (2009) used a technique similar to that of Seidler and Starr (1969) to obtain PI variants of *Bdellovibrio*. However, instead of selecting for streptomycin resistant PD *Bdellovibrio*, they cultured prey dependent *B. bacteriovorus* 109J on *E. coli* strain WM3064, a diaminopimelic acid auxotroph. Lastly, Tudor *et al*., (2008) isolated predation-deficient mutants of *Bdellovibrio* using random-transposon-insertion mutagenesis.

4.1.2. Genetics of prey-independent *Bdellovibrio* strains

The ability of *B. bacteriovorus* to grow in the absence of prey in a nutrient rich medium has been suggested to be as a result of spontenous single mutation (Seidler and Starr, 1969; Ishiguro, 1973; Varon and Seijffers, 1975; Friedberg, 1978). The first genetic locus associated with PI phenotype of *Bdellovibrio* was reported by Cotter and Thomashow, (1992). They observed that mutation at a particular region designated as host interaction (*hit*) locus is responsible for the

conversion of obligate predatory *B. bacteriovorus* 109J to PI phenotype. The *hit* locus was described as 959 bp *EcoRI-XbaI* fragment containing 4 open reading frames (ORFs) - ORF1, ORF2, ORF3 and ORF 4 (. The ORF1 and ORF2 were suggested to be part of the same operon because they overlap by 1 bp. The ORF2 is completely contained in the *EcoRI-XbaI* fragment, however, mutation in PI mutants described by Cotter and Thomashow affected ORF2, ORF3 and ORF4. The ORF2 is required for the *B. bacteriovorus* to produce large, clear plaque because it was fully restored when *hit* recombinant experiment was done by Cotter and Thomashow, (1992). The ORF2 encodes 10.6 kDa ORF2 hydrophilic polypeptide with signal peptide properties. Cotter and Thomashow, (1992) also suggested gene product of ORF2 as polypeptide with structural function or enzymatic activity needed to invade prey. Therefore, mutation at the *hit* will lead to poor prey penetration and poor plaque production. Meanwhile, ORF3 and ORF4 might contained the original spontaneous PI mutation, and maybe crucial in the PI phenotype because unlike ORF2, the loci were not restored or expressed in the *hit* recombinants. With the discovery of *hit* locus, Cotter and Thomashow, (1992) shed light on the molecular basis for the development of PI phenotype in *Bdellovibrio* as well as *Bdellovibrio*-prey interaction.

Barel and Jurkevitch, (2001) conducted a study on seven PI phenotypes isolated from the same wild type *B. bacteriovorus* 109J. They observed that only three out of the seven mutants had mutation at the *hit* locus, and therefore concluded that mutation at the *hit* region is not necessary for the development of PI phenotype but maybe one of the secondary mutations that can help *Bdellovibrio* to grow axenically. They further analyzed the ORFs of the *hit* locus as summarized below;

Table 4. 2. Putative ORFs detected in the hit locus of *Bdellovibrio bacteriovorus* 109J-1 (accession number AF368191)

pp ProteinPredict (http://www.embl-heidelberg.de/predictprotein/submit_def.html), ps PSORT (http://psort.nibb.ac.jp). (Barel and Jurkevitch, 2001)

Table 2 Putative ORFs detected in the *hit* locus of *Bdellovibrio bacteriovorus* 109J-1 (accession number AF368191). *pp* ProteinPredict (http://www.embl-heidelberg.de/predictprotein/submit_def.html), *ps* PSORT (http://psort.nibb.ac.jp)

	Start/stop	Domains	Signal peptide	Sequence similarity
ORF1	1/878[b]	Transmembrane (pp; ps)	Yes	None
ORF2[a]	312/7	Outer membrane (ps)	Yes	None
ORF3[a, c]	905 /333		No	*Bacillus subtilis* wall-associated protein precursor
ORF4[a]	215/1[b]		No	None

[a]Reverse strand
[b]Extends beyond the sequenced locus
[c]Nucleotide positions in strain HD100 (AJ401463)

In another experiment carried out by Roschanski *et al*., (2011), two types of mutants were described including Type I mutant that can grow saprophytically in the presence of prey cell extracts (autoclaved *E. coli* cells) without losing completely the predatory properties. And Type II mutant that grows axenically on nutrient-rich media not supplemented with prey cell extracts. All the 11 saprophytic PI mutants were reported to have experienced different types of mutations including nonsense or frameshift mutations affecting the putative gene products of a small ORF (*Bd0108*, termed *hit* gene) of the *hit* locus significantly. They further suggested that more mutations may occur apart from mutation from the *hit* locus during selection for the PI phenotype because whole genome sequencing of two mutants (M1 and M2) revealed the occurrence of four to five mutations including the one involving the *hit* gene. However, one of the mutants-M11/Strept only experience one frameshift mutation in the *hit* gene, suggesting that mutation in the *hit* gene alone is sufficient for the derivation of saprophytic PI. Furthermore, a mutation in two genes annotated as *Bd3461* (*rhlB*) – a part of degradosome involved in RNA degradation and a gene tagged *Bd3464* in *B. bacteriovorus* HD100 involved in RNA processing were observed to be responsible for axenic growth in the Type II mutants. The loss of function in RNA processing due to mutation in the degradasome results to an increase in the half-life of specific RNAs, which could be involved in the initiation of DNA replication thereby sending signals that can enhance colony formation in the axenic Type II mutants.

The works of Barel and Jurkevitch (2001) and Roschanski *et al*., (2011) showed that mutation in the *hit* locus is not the only condition that is needed for the development of *Bdellovibrio* PI phenotypes. The development of the PI phenotypes needs further studies to unravel other factors that play major roles in the process to derive PI phenotype. Furthermore, the possibility of obtaining PI phenotypes without mutation in the *hit* gene is an indication that the *hit* gene might not necessarily be playing a significant role in the derivation of PI phenotypes.

4.1.3. Description of *hit* locus and its adjacent genes

The genome of *B. bacteriovorus* HD100 published by Rendulic *et al*. in 2004 provided the opportunity to have an insight into the arrangement and positioning of the *hit* locus in *B. bacteriovorus* (Figure 4.2). The *hit* locus was described to be positioned downstream of putative pilus clusters which have ORFs that encodes a structural protein pilin of the Flp family; *flp1* and *flp2* usually involved in the invasion process. The high level of transcription of *hit* ORF and *flp1* in prey-seeking predators compared to the intraperiplasmic predators suggested that the gene products of *hit* (putative regulatory peptide) and *flp1* (structural protein for attachment and invasion) are important for attachment and invasion of prey during *Bdellovibrio* attack phase (Schwudke *et al*., 2005).

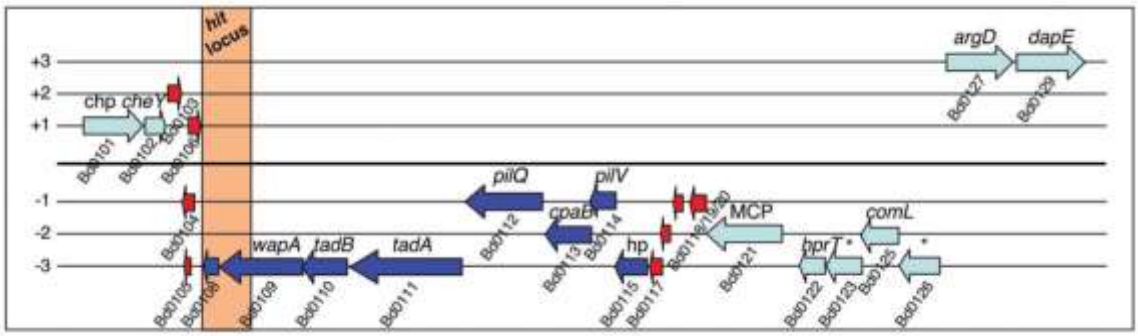


Figure 4. 2. The host interaction locus (*hit*) of *B. bacteriovorus*. The previously described *hit* locus (host interaction locus, orange bar) (9), consisting of a 950-bp sequence, is congruent with a section of the *B. bacteriovorus* genome that is predicted to code for pilus and adherence genes. The genes in this cluster are likely to functionally interact as structural elements of a pilus and are predicted to be transcribed as one transcriptional unit. The previously predicted gene *hit* corresponds to the ORF Bd0108 in the *B. bacteriovorus* HD100 genome and is part of the pilus and adherence transcriptional unit, together with a gene *wapA* coding for a cell wall–associated protein with a cellulose-binding domain (Bd0109), the flagellar pilus assembly genes tadA (Bd0111) and tadB (Bd0110), and additional pil genes of the type IV pilus. The *hit* locus containing the tad and pil gene cluster seems to have been inserted in between two chemotaxis genes (cheY, Bd0102; and mcp, Bd0121), because they are flanked on both sides by large potentially noncoding regions (red arrows) for which only short artificial or hypothetical ORFs could be found. The predicted ORFs of this region were assigned the following putative functions: chp, (conserved) hypothetical protein; WapA, cell wall–associated protein with a cellulose-binding domain; tad, tight adherence Flp pilus-assembly proteins TadB and TadA; pil, pilus assembly; cpaB, Flp pilus-assembly protein CpaB; MCP, methyl-accepting chemotaxis protein; hprT, hypoxanthine-guanine phosphoribosyltransferase; *, tetratrico peptide repeat domain–containing protein; comL, competence lipoprotein; argD, acetylornithine/succinyl-diaminopimelate aminotransferase; dapE, succinyl-diaminopimelate desuccinylase. **(**Rendulic *et al*., 2004)

A gene *bd0109* observed to be adjacent to the *hit* gene (*bd0108*) has been described by Capeness *et al*., (2013) to be important for the survival of PI and PD *Bdellovibrio* phenotypes. The *bd0109* and *bd0108* are co-transcribed, and interact with each other to regulate pilus extrusion in *Bdellovibrio* (Figure 4.3 and Figure 4.4). They further observed that a full deletion of the *bd0108* leads to the production of very few pilus fibres thereby causing a signal, promoting the axenic growth in *Bdellovibrio*. However, deletion of bd0109 gene does not lead to the development of axenic growth, thereby suggesting that it plays an essential role in the viability and growth of PI and PD *Bdellovibrio.* Furthermore, Prehna *et al*., (2014) used NMR to reveal *bd0108* as Intrisincally Disordered Protein (IDP). This findings further support the signaling and regulatory role of *bd0108* gene because IDP or intrinsically disordered regions have been reported to often participate in the regulation or signaling reactions in biological systems. The *hit* gene and the its adjacent genes with their respective functions are summarized in Table 4.2 below.

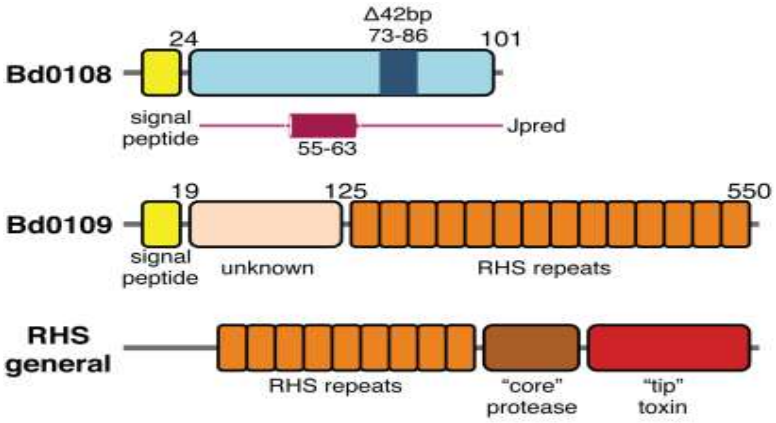
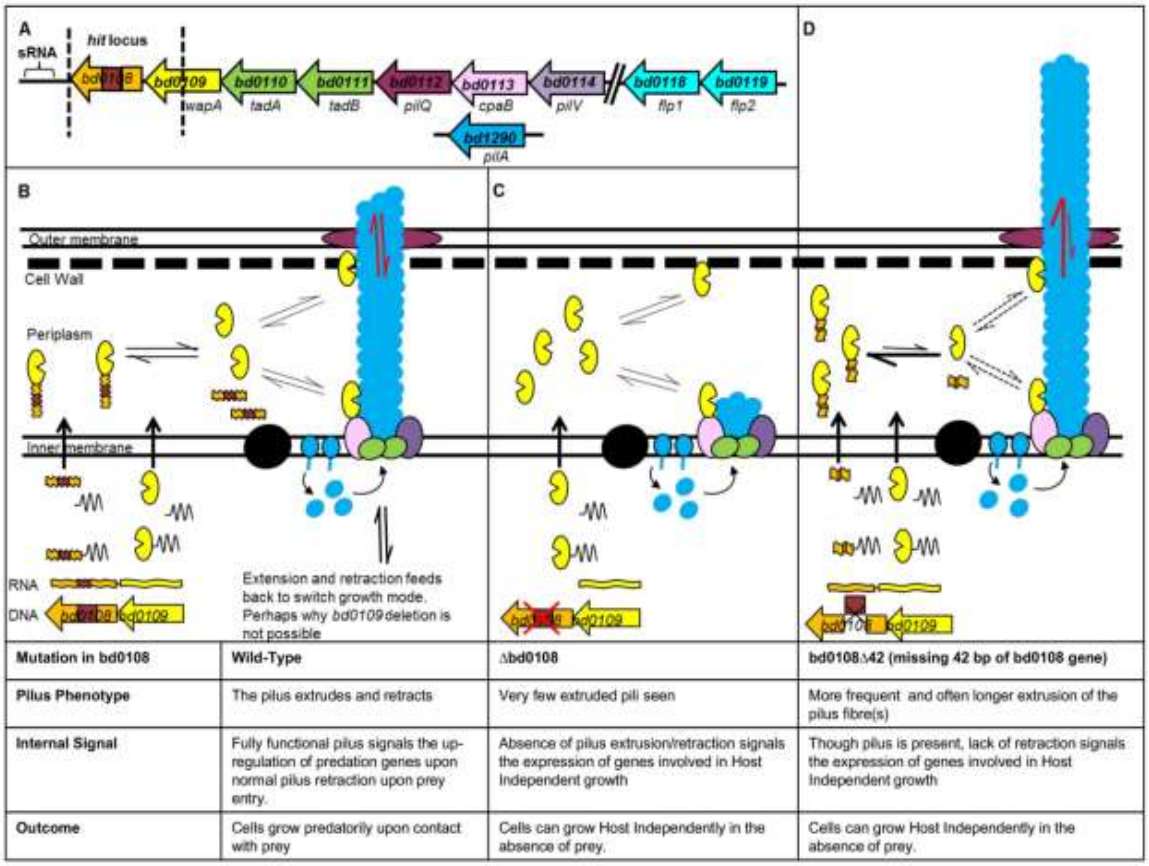


Figure 4. 3. Domain map of Bd0108 and Bd0109. Schematic of Bd0108 and Bd0109 based on sequence homology and structure prediction with a comparison to RHS family proteins. Bd0108 is annotated noting the region corresponding to the HI D42 bp deletion (above) and secondary structure prediction by Jpred (below). The magenta cylinder represents the predicted a-helix. Bd0109 is shown to consist of an N-terminal domain of unknown function and a C-terminal domain consisting of approximately 13 RHS repeat elements shown as orange boxes. The signal peptide for both Bd0108 and Bd0109 is displayed as a yellow box. RHS general represents a basic schematic for the domain organization of the RHS protein family. (Prehna *et al.,* 2014).



Mutation in bd0108	Wild-Type	Δbd0108	bd0108Δ42 (missing 42 bp of bd0108 gene)
Pilus Phenotype	The pilus extrudes and retracts	Very few extruded pili seen	More frequent and often longer extrusion of the pilus fibre(s)
Internal Signal	Fully functional pilus signals the up-regulation of predation genes upon normal pilus retraction upon prey entry.	Absence of pilus extrusion/retraction signals the expression of genes involved in Host Independent growth	Though pilus is present, lack of retraction signals the expression of genes involved in Host Independent growth
Outcome	Cells grow predatorily upon contact with prey	Cells can grow Host Independently in the absence of prey.	Cells can grow Host Independently in the absence of prey.

Figure 4. 4. Model for possible interactions of Bd0108/Bd0109 controlling the extrusion and retraction of pili.

A. Operonal structure of the bd0108 *hit* locus and surrounding genes, predicted to have a role in the formation of a Type IVb pilus. Genes are colour coded to correspond to their predicted function in the pilus diagrams underneath.

B. In wild-type cells bd0108 and bd0109 are co-expressed, the mRNA is then translated into proteins containing a signal sequence recognised by the Sec system, the signal is cleaved, and the proteins are transported into the periplasm where the mature Bd0108 protein transiently interacts with Bd0109 to sequester it. When Bd0109 is unbound, it could then anchor at either the cell wall, or with the mature pilus fibre. Both scenarios are possible due to Bd0109's structural cleft binding carbohydrate that is present in both cell wall and the mature and glycosylated pili. Bd0109 mediates successful pilus extrusion/retraction and signal back into the cytoplasm. In wild-type pilus formation Bd1290 pre-pilins are held in the inner membrane and are assembled into the pilus fibre possibly by the flp pilus ATPases Bd0110 and Bd0111. The balance of sequestering and release of Bd0109 by Bd0108 in the periplasm permits to successful extrusion and retraction of the pilus fibre upon environmental cues.

C. In the absence of Bd0108 protein, Bd0109 is not sequestered and is free to mediate more frequently with pilus extrusion and retraction, resulting in very few pili extruded beyond the cell surface and cues for HI growth signalled to the cell.

D. In HI strains containing the 42 bp deletion variant of bd0108, the gene is still expressed. The truncated form of Bd0108 alters the dynamics of the Bd0109 functionalisation are altered (possibly by over-sequestration of Bd0109) and hyper-extruded pili are seen on the surface more frequently. Hyper-extruded pili or no pili send similar internal signals to regulate prey independent growth. Capeness *et al*., (2013)

Table 4. 3. The *hit* gene and the its adjacent genes with their respective functions are summarized in Table below (Prehna *et al*., 2014)

Genes/Proteins	Description/Function
bd0110-bd0114,*bd0118*, *bd0119* and *bd1290*	Encode type IVb pilus needed for prey attachment and invasion in *Bdellovibrio*.
bd1290	The major core subunit and structural component of the pilus encoding a *PilA* homolog.
bd0118 and *bd0119*	Encodes major pilin homologs *flp1* and *flp2* respectively.
Bd0113 and Bd0114	Involved in the process of pili assembly
Bd0110 and Bd0111	Share similarity to TadA and TadB, which are ATPases that provide the energy for secretion. All four proteins localized to the predator's inner membrane
bd0112	shares homology with pilQ, which functions to both anchor the pilin structure in the outer membrane and to allow passage through the membrane
bd1509–1512, bd0867 (pilQ), bd1585 (pilM), bd2167 (pill) bd3852 (pilT)	putative Type IVa pilus in *B. bacteriovorus* HD100
Bd0108 and Bd0109	The proteins interact directly and work in concert to both promote the secretion of the *B. bacteriovorus* pilus and to regulate pilus length

4.1. MATERIALS AND METHODS

4.3. Isolation of prey-independent strains of *Bdellovibrio* according to method described by Ferguson *et al*., (2008).

This method was reported as a rapid method for the derivation of prey-independent *Bdellovibrio* phenotype from the predatory PD phenotype. Briefly, co-cultivation of *B. bacteriovorus* SSB218315 and *Bdellovibrio* sp. SKB1291214 with their respective prey–*Salmonella* sp. and *Klebsiella* sp. respectively was done in an HEPES buffer. The cultures were incubated at 30 ^{o}C for 5 days. After 5 days when clear lysis was observed, and microscopic examination has shown the presence of *Bdellovibrio*, the lysate was filtered through 0.45 µm syringe filter. A sterile 0.2 µm polycarbonate filter paper was placed on LB agar using sterile forceps. Thirty microliters of filtered lysate was spotted on the 0.2 µm polycarbonate filter paper and directly on the LB agar. The inoculated LB agar plate was then incubated at 22 ^{o}C and monitored for the characteristic yellow colony described for the PI *Bdellovibrio* phenotypes which appeared after 48 h. The yellow colonies that developed were allowed to increase in size and picked into LB broth. The LB broth was incubated at 22 ^{o}C and observed for turbidity which indicated bacterial growth. The broth culture was streaked on LB agar to obtain pure yellow colonies of the PI *Bdellovibrio* phenotypes. A colony from the pure yellow colonies on the LB agar was further inoculated into an LB broth and incubated at 22 ^{o}C. One milliliter (1 mL) of the LB broth bacterial culture was used for DNA extraction using Wizard® Genomic DNA purification kit according to manufacturer's instructions. The PCR amplification of 16S rDNA and *hit* locus was done using the primers described in Table 3 in objective 1 of this study.

4.4. Isolation of prey-independent strains of *Bdellovibrio* according to method described by Lambert and Sockett, (2008).

Co-cultivation of *Bdellovibrio* sp. strain SKB1291214 and prey was done in HEPES buffer and incubated until lysis was obtained. About 10 – 20 mL of lysate was filtered through 0.45 µm syringe filter. The filtrate was centrifuged at 7000 x g for 20 min at 4 ^{o}C. The supernatant was discarded and the resulting pellet was resuspended in 100 µL of Yeast-Peptone (YP) broth. The

pellet was then inoculated on the YP agar using spread plate technique. The plate was incubated at 30 ^{o}C for 3 days and observed for tiny slow-growing yellow colonies. A yellow colony obtained was subcultured in 50 mL YP broth and incubated at 30 ^{o}C with shaking at 200 rpm. Resultant growth was streaked on YP agar to obtain pure colonies. The DNA extraction and PCR amplification of 16S rDNA and *hit* locus were done using the primers described in Table 3.0 in objective 1 of this study.

4.5. Isolation of prey-indpendent strains of *Bdellovibrio* according to method described by Seidler and Starr, (1969).

4.5.1. Screening of Gram negative bacteria prey against streptomycin using disk diffusion and agar well diffusion method.

This method relied on the use of antibiotics (streptomycin) to selectively isolate prey-independent *Bdellovibrio* strains. Antibiotic susceptibility testing of streptomycin against eleven different Gram negative bacteria belonging to the member Enterobacteriaceae was done using both disk diffusion and agar-well diffusion method following the recommendations by Clinical Laboratory Standards Institute standards (CLSI, 2012). The plating medium was Mueller-Hinton agar (MHA). Briefly, five to eight colonies of the test organism from an 18 – 24 h old culture on LB agar was taken and inoculated into 3 mL of sterile physiological saline (0.85 % NaCl), mixed thoroughly and adjusted to give a turbidity equivalent 0.5 MacFarland standard (wavelength; 590 nm and optical density of 0.08 – 0.10). The bacterial suspension served as the inoculum for the antibiotic sensitivity resting. The inoculation of the Mueller-Hinton agar was done by inserting sterile cotton swab asceptically into the bacterial suspension. Excess fluid was removed by rotating the cotton swab (applicator) against the side of the test tube and the swab was applied on the entire surface of the Mueller-Hinton agar. For disk diffusion susceptibility testing method, streptomycin disc (10 µg) was placed on the surface of the inoculated Mueller-Hinton agar plates using a pair of sterile forceps. For agar-well diffusion susceptibility testing method, a sterile cork borer was used to punch two holes of about 6-8 mm diameter into the Mueller-Hinton agar, and 30 µL of 250 µg/mL and 500 µg/mL of streptomycin was introduced into each separate well using sterile pipette tips.

The plates were left on the laboratory bench for about 5 minutes to allow the antibiotics diffuse into the media and incubated at 37 ^{o}C for 24 h. The results of the diameters of zone of inhibition were interpreted by comparing it with the Clinical Laboratory Standards Institute standards (CLSI, 2012) and the isolate was recorded as resistant, intermediate or susceptible to streptomycin. The following strains were selected for further work: streptomycin-resistant *E. coli* 3A and streptomycin-sensitive *Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251)

4.5.2. Isolation of prey-independent *Bdellovibrio* strains.

Prey dependent *Bacteriovorax* sp. SSFD2 was cultured with streptomycin-resistant *E. coli* 3A in HEPES buffer medium to derive prey-independent streptomycin resistant strain designated PI-BVX^{smr}. The culture was incubated overnight at 30 ^{o}C. Then, 20 mL of the overnight culture was transferred into an HEPES buffer medium containing streptomycin (500 µg/mL) and streptomycin-resistant *E. coli* 3A. The culture was incubated at 30 ^{o}C for 7 days and observed for lysis. The lysate was washed off streptomycin by centrifuged at 3,000 rpm for 20 min at 4 ^{o}C, filtered using 0.45 µm syringe filter and resultant prey dependent streptomycin resistant PI-BVX^{smr} in the filtrate was cultured with streptomycin-sensitive *Salmonella* enterica subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251) in HEPES buffer. The culture was incubated at 30 ^{o}C for 7 days and observed for lysis. The lysate obtained was centrifuged at 3,000 rpm for 20 min at 4 ^{o}C, filtered using 0.45 µm syringe filter. The filtrate was observed under the light microscope (Olympus U-TVO.35XC-2, T2 Tokyo, Japan). A portion, about 10 mL of the resultant filtrate was centrifuged at 7,000 x g for 20 min at 4 ^{o}C. The resulting pellet was resuspended in 100 µL of HEPES buffer and cultured on a selection medium which is YP agar supplemented with 500 µg/mL of streptomycin using spread plate technique. Another portion of lysate, about 10 mL was transferred into YP broth supplemented with 500 µg/mL of streptomycin. The agar plate and broth were incubated at 30 ^{o}C for 7 days and observed for bacterial growth (formation of yellow colony on plate and turbidity in the broth). Bacterial colonies obtained were streaked on YP agar plate supplemented with 500 µg/mL of streptomycin to obtain pure colonies. The DNA extraction and PCR amplification of 16S rDNA and *hit* locus were done using the primers described in Table 3 in objective 1 of this study.

4.6. Gram stain reaction

Gram stain technique is a differential staining procedure that separate bacteria into two classes: Gram-positive and Gram-negative. The method was developed in 1884 by the Danish physician Christian Gram. A smear of 18 – 24 h old culture on LB or YP agar was prepared on a clean microscope slide. The smear was then heat-fixed by passing the slide through a Bunsen burner flame. The smear was flooded with crystal violet and allowed to react for one minute after which the stain was poured off and the smear rinsed under gentle running tap water. Thereafter, the slide was flooded with Gram' s iodine (a mordant) and then rinsed off under gentle running tap water. The smear was later decolourized with 95 % ethanol, rinsed under gentle running tap water, and counter-stained with safranin for about thirty seconds. The slide was then washed under gentle running water, allowed to air-dry and then examined under the oil immersion objective of the light compound microscope (Olympus U-TVO.35XC-2, T2 Tokyo, Japan).Gram-positive bacteria appeared as purple in colour while Gram-negative bacteria appeared as pink.

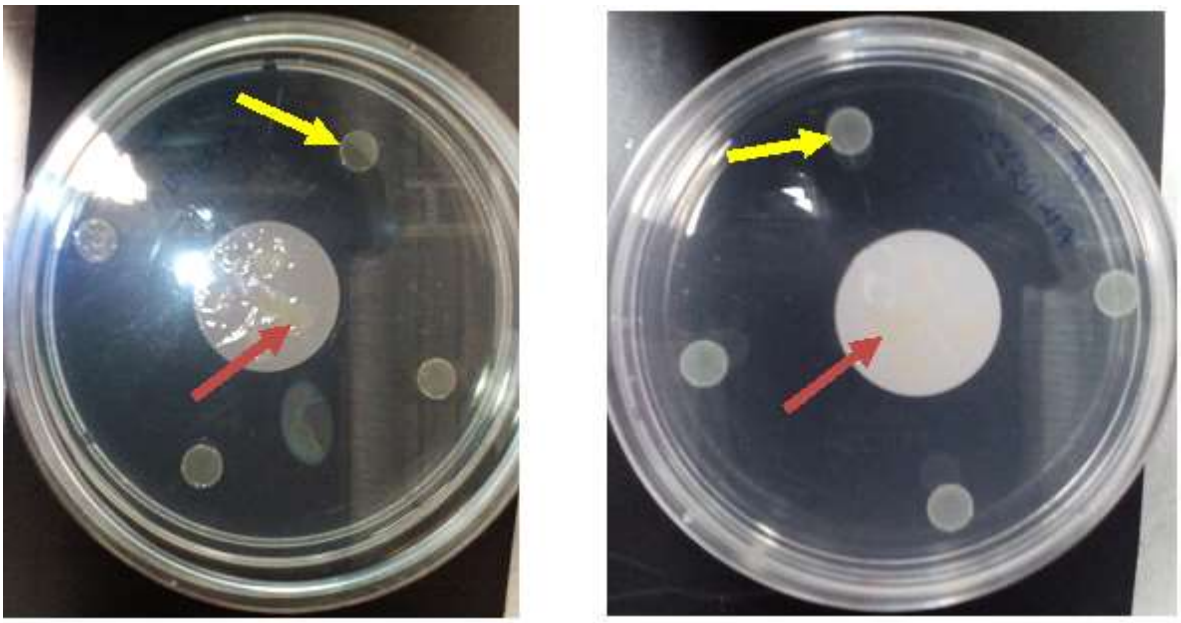
4.7. Molecular identification of prey-independent *Bdellovibrio* strain

Pure colonies of PI strains were cultured in YP broth 30 ^{o}C for 48 h. Genomic DNA was extracted using Wizard® Genomic DNA purification kit according to manufacturer's instructions. The 16S rRNA gene was amplified using the following primers BbsF216 and BbsR707, and the for the *hit* gene amplification, the primers BdhitF and BdhitR were used (Table 3). In addition, universal primers 16S-F: 5' AGAGTTTGATCCTGGCTCCAG 3' (*E.coli* location 8-28) and 16S-R: 5' ACGGCTACCTTGTTACGACTT 3' (*E. coli* location 1492) that amplified a 1533 bp fragment of the 16S rRNA gene was also used. The amplified 16S rRNA gene PCR products obtained were purified using QIAquick Gel extraction kit (Hilden, Germany) and sequenced by Eurofins MWG Operon© LLC company (www.operon.com; 2211 Seminole Drive Huntsville, Alabama 35805, USA).

RESULTS

4.8. Isolation of prey-independent strains of *Bdellovibrio* according to method described by Ferguson *et al*., (2008) and Lambert and Sockett (2008)

Based on the methodology described by Ferguson *et al*., 2008 and Lambert and Sockett, 2008, the slow-growing yellow colonies similar to what was reported earlier in literature was obtained when the lysate was spotted on 0.22 µm polycarbonate filter paper, LB agar (Ferguson *et al*., 2008), Figure 4.5A & B, and spread on YP agar (Lambert and Sockett, 2008). The phenotypic characteristics exhibited by the pure colonies of these suspected PI-*Bdellovibrio* strains (Figure 4.6 A, B and 4.7 C) were observed to be identical as reported earlier; yellow, rod-shaped, Gram-negative bacteria (Figure 4.7 B).

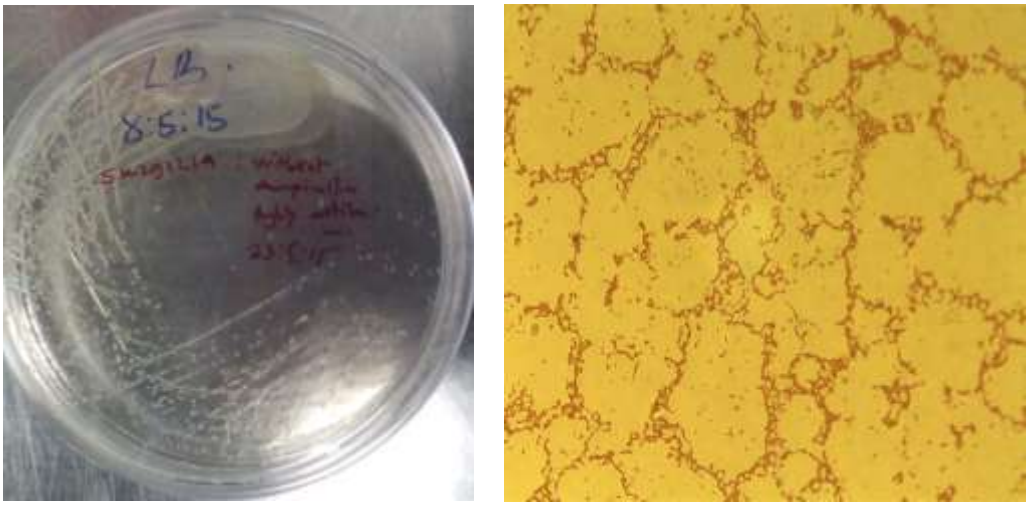


A. Suspected PI SSB218315 B. Suspected PI SKB1291214

Figure 4. 5. (A). Suspected yellow prey-independent colonies derived from prey-dependent. *Bdellovibrio* spp. SSB218315 and (B). SKB1291214 growing on 0.22 µm polycarbonate filter paper laid on LB agar (red arrow) and directly on LB agar (yellow arrow).

A **B.**

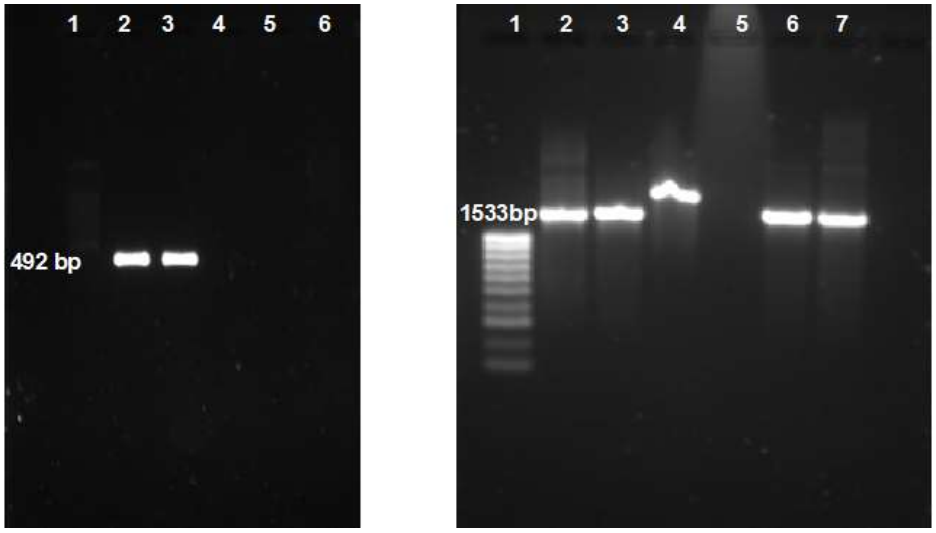
Figure 4. 6 (A). Pure tiny yellow colonies of suspected PI *Bdellovibrio* spp. SKB1291214 obtained as described by Lamber and Sockett (2008) and (B). SSB218315 obtained as described by Ferguson *et al*., 2008 growing on YP and LB agar respectively.



A. **B.**

Figure 4. 7. (A). Pure tiny yellow colonies of suspected PI *Bdellovibrio* spp. SKB1291214 obtained as described by Ferguson *et al*., 2008 growing LB agar. (B). Gram stain reaction of one of the suspected PI-*Bdellovibrio* strain showing it as Gram-negative rods.

The PCR amplification of the 16S rRNA gene to confirm the pure bacterial isolates as PI-*Bdellovibrio* using *Bdellovibrio*-specific primer that amplified 492 bp of the 16S rRNA gene fragment was negative (Figure 4. 8A and B). However, amplification of the 16S rRNA gene using universal primer was successful. The BLAST analysis of the sequences obtained from the amplified PCR product showed identity with *Microbacterium* spp. instead of *Bdellovibrio* spp. Furthermore with the isolation procedure described by Ferguson *et al*., (2008), and Lambert and Sockett (2008), It was observed that there is the tendency of residual prey present after filtration to interfere with the development of pure prey-independent *Bdellovibrio*. Therefore, another method described by Seidler and Starr (1969) was used for comparison.



A. **B.**

Figure 4. 8. (A). Agarose gel image of the PCR amplification for the identification of suspected PI *Bdellovibrio* spp. SKB1291214 and SSB218315. *Bdellovibrio*-specific primer was used to amplify 492 bp of the 16S rRNA gene fragment with prey depedent (PD) *B. bacteriovorus* SSB218315 and SKB1291214 serving as positive control. [1.] 100bp Ladder; [2.] SKB1291214; [3.] SSB218315; [4.] PI-SK291214; [5.] PI-SSB218314; [6.] sterile milliQ wáter. (B). Agarose gel image of the PCR amplification for the identification of suspected PI *Bdellovibrio* spp. SKB1291214 and SSB218315. A universal primer was used to amplify 1533 bp of the 16S rRNA gene fragment using *Bdellovibrio* sp. SKB1291214 as positive control. [1.] 100 bp Ladder; [2.] PD-HD100; [3.] PD-SSB218315; [4.] PD-SKB1291214; [5.] sterile milliQ wáter; [6]. PI-SKB1291214; [7]. PI-SSB218315.

4.9. Isolation of prey-independent strains of *Bdellovibrio* according to method described by Seidler and Starr., (1962).

This method is more reliable when compared with the other two procedures earlier described because prey that can affect the growth of PI-*Bdellovibrio* would have been eliminated by the streptomycin, and the suspected PI-*Bdellovibrio* strains grew distinctly on the YP agar.

4.9.1. Screening of Gram-negative bacteria prey against streptomycin using disk diffusion and agar well diffusion method.

Rapid screening of eleven different bacterial strains for antibiotic susceptibility to 10 µg (antibiotic disc), 250 µg/mL and 500 µg/mL of streptomycin was done (Figure 4. 9A and B, Table 4.3 and 4.4). Finally, *Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251) was selected as the susceptible strain while *E. coli* 3A was taken as the resistant strain.

A. **B.**

Figure 4. 9. The result of the antibiotic susceptibility testing of streptomycin against *Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251) and *E. coli* 3A using disc diffusion and agar-well diffusion method. *Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251) (A) was susceptible while *E. coli* 3A was resistant (B).

Table 4. 4. The result of the antibiotic susceptibility testing of streptomycin (10 µg, 250 µg/mL and 500 µg/mL) against 11 different bacterial strains belonging to the member Enterobacteriaceae

Bacterial strains	**Diameter**		
	10 µg (mm)	**250 µg/mL (mm)**	**500 µg/mL (mm)**
E. coli 1[1]	11 (R)	15	15
Serratia marcescens CDBB-B-1014 (ATCC 14756)[2]	8 (R)	9	11
**E. coli* 3A[1]	6 (R)	10	10
E. coli CDBB-B-1107 (ATCC 8739)[2]	12 (I)	16	17
E. coli 5B[1]	12 (I)	12	15
Salmonella sp. D[1]	15 (S)	11	15
Salmonella sp. C[1]	12 (I)	13	15
Salmonella sp. B[1]	12 (I)	12	15
***Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251)[2]	13 (I)	16	19
Klebsiella sp.[1]	12(I)	11	16
Klebsiella oxytoca B-968 (ATCC 13182)[2]	9 (R)	13	15

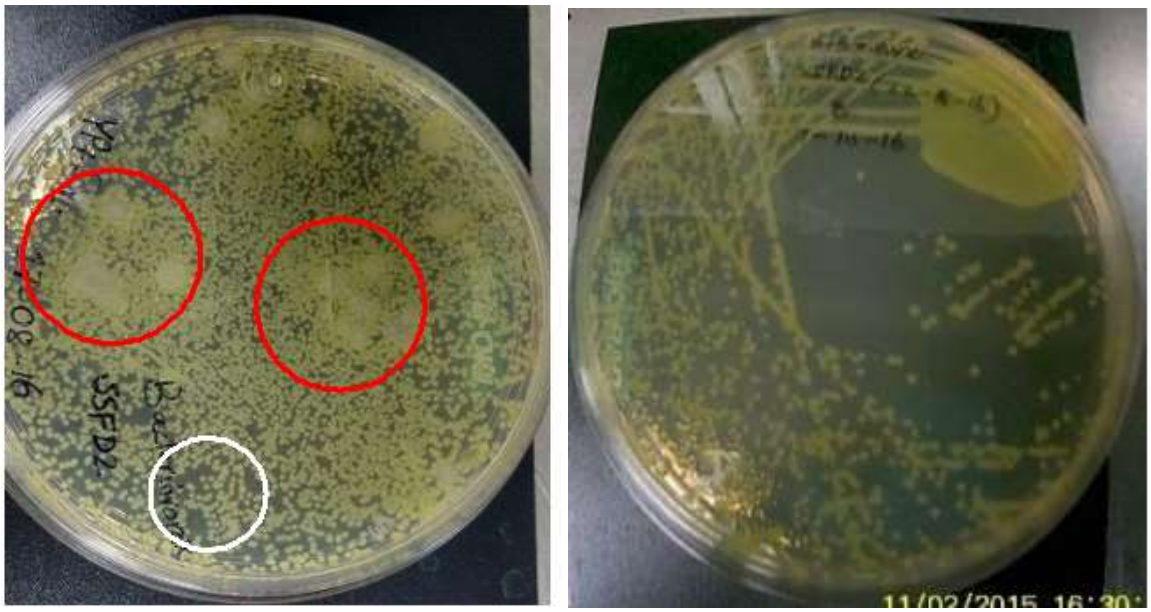
[1]Reference bacterial isolates obtained from National Collection of Microbial Strains and Cell Culture of CINVESTAV., [2]laboratory bacterial strains obtained from Center for Genomic Biotechnology (IPN), *Selected streptomycin resistant bacterium for the isolation of prey-independent *Bdellovibrio* strains, ** Selected streptomycin susceptible bacterium for the isolation of prey-independent *Bdellovibrio* strains, R = Resistant, I = Intermediate, S = Susceptible.

Table 4.4. Zone diameter (mm) interpretative standards for Enterobacteriaceae according to Clinical Laboratory Standard Institute (CLSI, 2012)

Antibiotic (Disc concentration)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Streptomycin (10 µg)	≤ 11	12 - 14	≥15

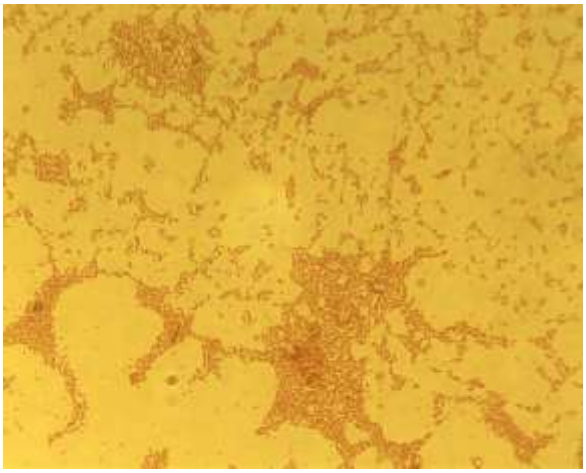
4.9.2. Isolation of prey-independent *Bdellovibrio* strains

The results obtained using method described by Seidler and Starr (1969) was similar to the results obtained when protocol described by Ferguson *et al*., (2008), and Lambert and Sockett (2008) were used. The cultivation of prey dependent strain with streptomycin resistant prey (*E. coli* 3A) in the presence of streptomycin, selected for the growth of streptomycin resistant prey dependent strains (PD-BVX^{smr}). When the PD-BVX^{smr} was cultured in YP broth containing streptomycin after co-cultured with the streptomycin susceptible prey (*Salmonella enterica* subsp. Enterica serovar typhi CDBB-B-1101 (ATCC 7251), microscopic examination revealed the presence of dead prey cells and highly motile BVX-PDSm^{R}. The cultivation of PD-BVX^{smr} on YP agar supplemented with 500 µg of streptomycin (selective medium) resulted into the growth of tiny yellow colonies (suspected PI-BVX^{smr}) that increased in sizes with increased days of incubation (Figure 4. 10A, indicated by the white circle). Furthermore, among this yellow colonies are patches of *Salmonella* prey that survived the effect of the streptomycin (Figure 4. 10A, indicated by the red circles). The colonies were then subcultured on fresh YP agar to obtain pure colonies. The phenotypes of the suspected PI-BVX^{smr} are Gram-negative rod-shaped yellow colonies (Figure 4. 10 A, B and C). Furthermore, the intensity of the yellow colour of the colonies was observed to reduce upon subsequent subculturing (Figure 4. 11 A and B)..

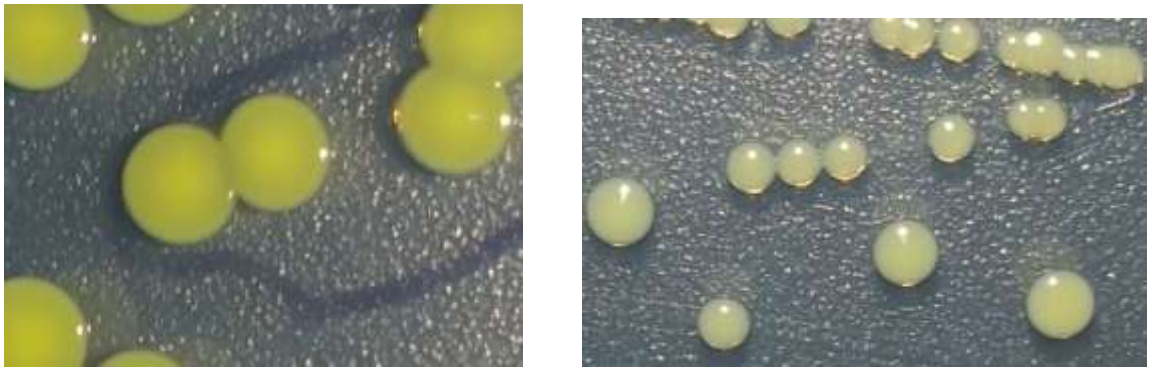


A. B.

Figure 4. 10. A. Suspected prey-independent streptomycin resistant strain (PI-BVX^{smr}) growing as yellow colonies on YP agar supplemented with 500 µg of streptomycin. This colonies were derived when prey dependent strain designated *Bacteriovorax* sp. SSFD2 was cultured using isolation protocol described by Seidler and Starr, (1969). B. Pure yellow colonies of suspected prey-independent strain grown on YP agar.



(C). Gram stain reaction of one of the suspected PI-strain designated BVX showing it as Gram negative rods.



A. High intensity yellow colour of colonies B. Colonies with reduce intensity of the yellow colour

Figure 4. 11. Enlarged images of the PI-BVX showing the reduction in the intensity of the yellow colonies upon subsequent subculturing.

4. 10. Molecular identification of prey-independent *Bdellovibrio* strain

The PCR amplification of 16S rDNA for three randomly selected yellow colonies from the plate shown in Figure 4. 10A i.e colonies obtained before purification of colonies by streaking on fresh YP agar was initially positive (Figure 4. 12). However, when the yellow colonies were streaked on another YP agar plate for purification, the amplification of the 16S rDNA was subsequently negative (result not shown). The negative result obtained by PCR amplification of the 16S rRNA gene prompted the use of the universal primer described above that amplified general bacterial 16S rRNA gene. PCR amplification was positive for the three bacterial isolates (Figure 4. 13). However, the sequencing result showed (Figure 4. 14) the bacterial isolates to share identity with *Microbacterium* sp. instead of prey-independent *Bdellovibrio* phenotypes as observed when the protocols of Ferguson *et al*., 2008 and Lambert and Sockett, 2008 was used. Furthermore, the PCR amplification of the *hit* locus was also negative for all the suspected prey-independent phenotypes (Figure 4. 15). The results obtained was the same for all the methodologies employed in obtaining the prey-independent phenotypes.

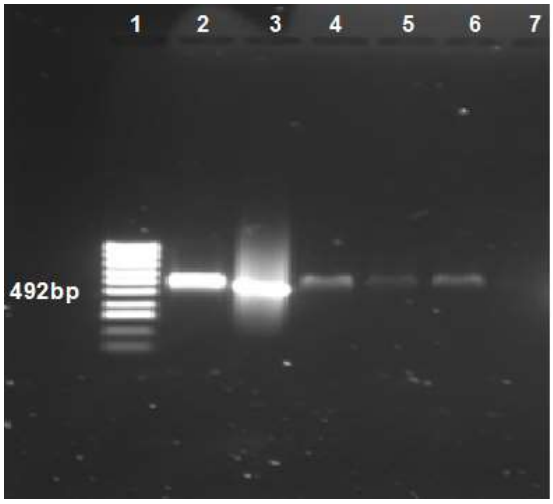


Figure 4. 12. PCR amplification of the first isolated yellow bacterial colonies before obtaining pure yellow colonies using 16S rDNA primers that amplified 492 bp fragment of the 16S rRNA gene. [1]. 100bp Ladder. [2]. Prey dependent *Bacteriovorax* sp. SSFD2. [3]. Prey dependent *Bdellovibrio* sp. SSB218315. [4]. prey-independent BVX1. [5]. Prey-independent BVX2. [6]. Prey-independent BVX3. [7]. Sterile milliQ water.

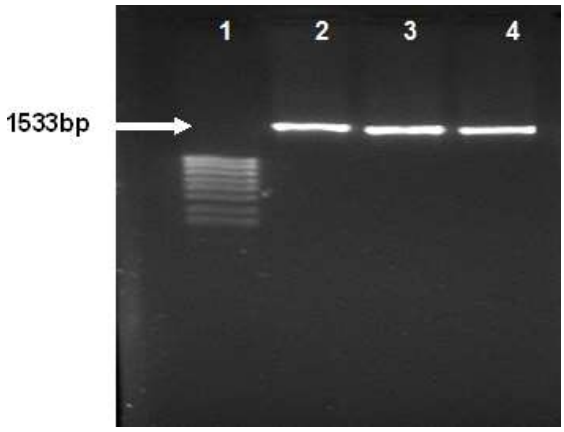


Figure 4. 13. PCR amplification of the 16S rRNA gene of the pure yellow bacterial colonies using universal 16S rRNA primers that amplified 1533bp fragment of the 16S rRNA gene [1]. 1kb Ladder [2]. Prey-independent BVX1 [3]. prey-independent BVX2. [4]. prey-independent BVX3.

>16S rRNA gene sequence for suspected prey-independent generated using universal 16S rRNA primer and SKB1291214 (1148 bp) sharing identity with *Microbacterium* spp.

NNNNNNNNNNTTGCNNNNACTGCAGTCGNCGGTGAACACGGAGCTTGCTCTGTGGGATCAGTGGCGAACGG
GTGAGTAACACGTGAGCAACCTGCCCCTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATACTGGATAT
GTGACGTGACCGCATGGTCTGCGTTTGGAAAGATTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTG
TTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCA
GCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACG
GTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTC
GGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAAT
GCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAAGAGCGAAA
GGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTC
CATTCCACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCNNAAGGCNAAA
CTCAAAGGAATTGACGGGGACCNNACAAGCGGCGGAGNNNNNGGATTAATTCGATGCACCNNAAANNCCTT
ACCAGGCTTGACTTNNNNANAANGGNCCAAAATGGTCACTCTTNGGANNNNTAANCANNNNNNNNGGTNNC
CNNCCCNNNNNNNGANNNNNNNNNNNNCCCNAACAAGCCACCNNNNNNTTTTNCCNNNNNNNNNNGNNCCT
TGGNAANNCNGGTTANNNNNNGAAGGGGNAANCNNNANNNNNGGCNNNNNNNGGNTNNNNNNNANTNNNGN
NNNNNNGNTCAA

>16S rRNA gene sequence for prey-independent BVX (394 bp) generated using universal 16S rRNA primer and sharing identity with *Microbacterium* spp.

GNNNNNNNNNNNNCTNNCNNGCNNTCNAACGGTGAACACGGANCTTGCTCTGTGGGATCAGTGGCAAACGG
GTGAGTAACACGTGAGCAACCTGCCCCTGACTCTGGGATAACCGCTGGAAACGGCGTCTAATACTGGATAT
GTGACGTGACCGCATGGTCTGCTTTTGGAAAAATTTTTCGGTTGGGGATGGGCTCGCGGCCTATCACCTTG
TTGGTGAGGTAATGGCTCACCAAGGCGTCAACGGGTAGCCGGCCTGAAAGGGTGACCGGCCACACTGGGAC
TGAAACACGGCCCAAACTCCTACGGGAGGCAGCATTGGGGAATATTGCACAATGGGCGGAACCCTGATGCA
CCACCCCCCCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTACCAGGAAAAAAGCAAAAGTGACG
GTACCTGCAAAAAAAGCCCCGGCTAACTACGTGCCACCACCCGCGGTAATACGTAGGGCGCAAGCGTTTTC
CGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCNCGTCTGCTGTCAAATCCCA

>16S rRNA gene sequence for prey-independent SCRB3 (556 bp) generated using universal 16S rRNA primer and sharing identity with *Microbacterium* spp.

GNNNNNNNNNNNNCTNNCNNGCNNTCNAACGGTGAACACGGANCTTGCTCTGTGGGATCAGTGGCAAACGG
GTGAGTAACACGTGAGCAACCTGCCCCTGACTCTGGGATAACCGCTGGAAACGGCGTCTAATACTGGATAT
GTGACGTGACCGCATGGTCTGCTTTTGGAAAAATTTTTCGGTTGGGGATGGGCTCGCGGCCTATCACCTTG
TTGGTGAGGTAATGGCTCACCAAGGCGTCAACGGGTAGCCGGCCTGAAAGGGTGACCGGCCACACTGGGAC
TGAAACACGGCCCAAACTCCTACGGGAGGCAGCATTGGGGAATATTGCACAATGGGCGGAACCCTGATGCA
CCACCCCCCCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTACCAGGAAAAAAGCAAAAGTGACG
GTACCTGCAAAAAAAGCCCCGGCTAACTACGTGCCACCACCCGCGGTAATACGTAGGGCGCAAGCGTTTTC
CGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCNCGTCTGCTGTCAAATCCCA

Figure 4. 14. The generated sequence for PI-*Bdellovibrio* strains using universal 16S rRNA primer

>16S rRNA gene sequence for prey-independent SSB218315 (718 bp) generated using universal 16S rRNA primer and sharing identity with *Microbacterium* spp.

NNNNNNNNNNNNGCTTACNNTGCAAGTCGAACGGTGAACACGGAGCTTGCTCTGTGGGATCAGTGGCGAAC
GGGTGAGTAACACGTGAGCAACCTGCCCCTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATACTGGAT
ATGTGACGTGACCGCATGGTCTGCGTTTGGAAAGATTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCT
TGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATG
CAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGA
CGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACC
TCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAACGGTGGA
ATGCNCAGATATCAGGAGGANNACCCATGGCNNNGGCAAATCTCTGGCCNANNNCNNNNTCTCNNNAACCA
AAAGGGNN

Figure 4. 14. The generated sequences for PI-*Bdellovibrio* strains using universal 16S rRNA primer

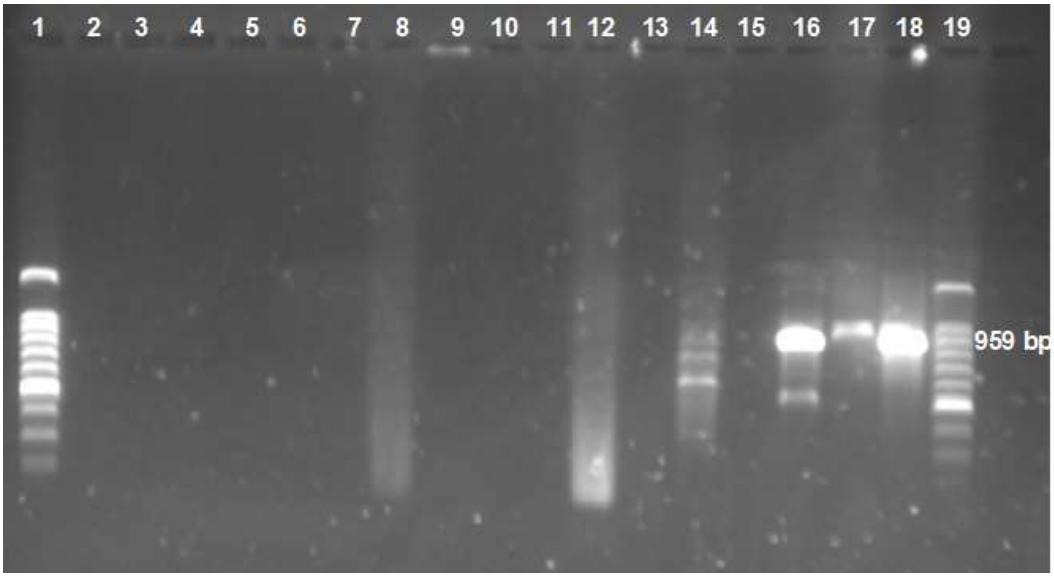


Figure 4. 15. **PCR** amplification of the *hit* locus of the pure yellow bacterial colonies using primers that amplified 959 bp fragment of the *hit* locus.

[1]. 100bp Ladder. [2]. Prey-independent SKB (14-12-15). [3]. Prey-independent BVX3 (22-8-16) [4]. prey-independent SSB (26-8-16). [5]. Prey-independent SSB (4-1-16). [6]. Prey-independent BVX1 (22-8-16). [7]. Prey-independent BVX2 (22-8-16) [8]. Prey-independent SKB (29-05-15) [9]. Prey-independent SKB (1-1-15). [10]. Prey-independent SKB (14-12-15) [11]. Prey-independent SKB (4-1-16). [12]. HIBA [13]. HIBB. [14.] Prey-independent SKB (31-1-15). [15]. Sterile milliQ water. [16]. Prey-dependent HD100. [17]. Prey-depenedent SCRB3. [18]. Prey-dependent SSB218315. [19]. 100 bp Ladder.

DISCUSSION

Prey-independent derivatives of obligate predatory *Bdellovibrio* spp. that are capable of growing on nutrient rich media in the absence of prey have been reported in several publications. These prey-independent derivatives were derived in the laboratory using different techniques except *B. bacteriovorus* strain Tiberius that was described to grow simultaneously both in the presence and absence of prey. These prey-independent strains or derivatives exhibit different forms of phenotypes ranging from colour differences (yellow to whitish grey), pleomorphism with morphology ranging from comma-shape to spiral shape and including some derivatives losing their motility and predacity. In addition, the *hit* locus that is associated with the development of the prey-independent phenotypes are found to be intact – without mutations in some prey- independent derivatives.

In this study, bacteria that exhibited similar phenotypic traits described for prey-independent derivatives of *Bdellovibrio* spp. were isolated from the prey-dependent isolated in the objective 1 of this study. The three methods used to isolate these prey-independent phenotypes gave the same result, however, the method of isolation described by Seidler and Starr, (1969) was more reliable.The prey-independent strains obtained in this study grew slowly on YP and LB medium, with tiny colonies appearing after 48 h. The tiny colonies increased in size to form colonies with yellow pigments. The formation of yellow pigment by *B. bacteriovorus* HD100 has been described to be as a result of the production of carotenoids. Moreover, genes associated with carotenoid synthesis (phytoene) has been reported to be present in *B. bacteriovorus* HD100 (bd1723-bd1725 and bd1730) (Hobley *et al*., 2012). Moreover, genome analysis carried out in the objective three of this research work revealed the presence of similar genes in *Bdellovibrio* sp. SKB1291214 (accession number OWT44676.1) and *B. bacteriovorus* SSB218315 (B9G79_RS10355 and B9G79_RS10360). During the periplasmic stage, the carotenoid protects *Bdellovibrio* from oxidative damages that may arise due to free radicals (Lambert *et al*., 2010). Furthermore, when the subsequent subculturing of the yellow colonies obtained in this study was done, the intensity of the yellow colour of the derivatives was observed to reduce (Figure 4. 9). The ability of the intensity of the yellow colour of prey-independent derivatives to reduce with successive

transfer has been reported by Seidler and Starr (1969). This has been attributed to a decrease in pigment produced by these prey-independent derivatives.

A wet mount microscopic examination showed that these strains are pleomorphic as described in different studies (Seidler and Starr, 1969; Barel and Jurkevitch, 2001) with a mixture of spiral, long and small rod shaped bacteria. Some of the bacteria were also observed to be motile under the microscope while some were not. Gram stain reaction further showed that the isolated yellow bacteria are Gram-negative.

The bacteria isolated as prey-independent in this study exhibited the phenotypic characteristics described for all reported prey-independent *Bdellovibrio*. Medina *et al*., (2008) reported to have confirm the prey-independent isolated in their studies by PCR amplification of 16S rRNA and *hit* locus using Bdellovibrionaceae-specific primers. Most publications used PCR amplification of *hit* locus to confirm the prey-independent derivatives (Dashiff and Kadouri, 2009; Ferguson *et al*., 2008). In this study, PCR amplification of the 16S rRNA gene in three randomly selected yellow colonies of suspected prey-independent *Bdellovibrio* phenotypes obtained before subculturing using *Bdellovibrio*-specific primer that amplified the 492 bp fragment of the 16S rRNA was initially successful. However, after subculturing the suspected prey-independent *Bdellovibrio* by transferring onto a fresh YP agar, the PCR amplification of the 16S rRNA and *hit* locus using *Bdellovibrio*-specific became negative. This therefore prompted the use of universal 16S rRNA primer to confirm the prey-independent *Bdellovibrio* derivatives. Amplified products were obtained for the universal primer but BLASTn analysis of nucleotides obtained after sequencing of the amplified PCR products showed that the derived yellow bacteria obtained from the prey-dependent *Bdellovibrio* shared identity with *Microbacterium* spp. with significant e-value (0.0) and percentage identity. Though several studies have reported the successful isolation of prey-independent derivative of *Bdellovibrio* in the laboratory, however, it is logical to hypothesize that the initial successful PCR amplification of the 16S rRNA obtained in this study might be due to the presence of some prey-dependent *Bdellovibrio* residue in the YP agar. This prey-dependent residue might be surviving on prey cells or prey cell extracts present in the filtrate that was spread on the YP agar.

And after subculturing by transferring a colony of the yellow colonies into a YP broth, the prey-dependent *Bdellovibrio* could not survive giving rise to the development of yellow coloured *Microbacterium* sp. on the YP agar.

Microbacterium spp. are Gram-positive (unlike *Bdellovibrio* spp. that are Gram-negative), slender, irregular, non-motile rod-shaped bacteria with a size ranging between 0.4 – 0.8 µm by 1.0 – 4.0 µm. They belong to the class Actinobacteria, and also capable of producing yellow pigmentation. Some strains have been reported to have the ability to degrade hydrocarbon (Manickam *et al*., 2006) and produce keratinolytic protease (Thys *et al*., 2004) while some strains have been implicated in bacteremia (Laffineur *et al*., 2003).

The relationship that exist between *Bdellovibrio* spp. and *Microbacterium* spp. can also come to mind with the results obtained. Also, comparing the size of *Microbacterium* spp. with *Bdellovibrio* spp. (0.2 – 0.5µm by 0.5 – 2.5 µm), few *Microbacterium* spp. might have passed through the 0.45 µm syring filter to contaminate or interfere with the growth of the prey-independent *Bdellovibrio* derivatives. In addition, a case of molecular misidentification with the universal 16S rRNA gene primers could not be ruled out in explaining the disparity observed in the phenotypic and molecular identification of the suspected prey-independnet *Bdellovibrio* spp.

Similar to the result obtained with the amplification of the 16S rRNA gene, PCR amplification of the *hit* locus for all the yellow colonies were negative. It has been reported that mutation in the *hit* locus is responsible for the development of prey-independent derivatives (Cotter and Thomashow, 1992). Though some prey-independent strains without mutation at this *hit* locus has been reported (Ferguson *et al*., 2008; Dashiff and Kadouri, 2009). The negative PCR amplification might be as result of mutation of the *hit* locus in the yellow colonies obtained in this study or because *Microbacterium* spp. does not have the *hit* locus.

The results obtained for this objective is inconclusive. A comparison between the suspected prey-independent phenotypes obtained in this study with referenced strains of prey-independent *Bdellovibrio* obtained from culture collection center or scientists in this field of research

will further elucidate the results obtained in this study. The use of more advanced identifiication techniques such as Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) can assist in confirming the identified suspected prey-independent derived phenotypes. Finally, the biochemical characteristic of the suspected prey-independent derivatives could not be done because of the inability to confirm the derivatives as *Bdellovibrio* spp.

CHAPTER FIVE

Objective 3: To carry out whole genome sequencing and analysis of selected isolated *Bdellovibrio* strains

5.1. INTRODUCTION AND LITERATURE REVIEW

5.1.1. Genomic studies on Bdellovibrio bacteriovorus

The complete genome of *B. bacteriovorus* strain HD100 was first reported in 2004 (Rendulic *et al*., 2004). *B. bacteriovorus* HD100 was reported to have large genome size of 3, 782, 950 bp which codes for 3584 proteins. Among these are hydrolytic enzymes- proteases and peptidases, glycanases, deoxyribonucleases (DNases), ribonucleases (RNases), and lipases needed by *B. bacteriovorus* at different stages of infection cycle including invasion of prey cells, digestion of prey cellular materials and escape from bdelloplasts or dead prey cells (Table 5.0). The genome also codes for motility and flagellar synthesis genes and flagellins. There are many *pil* genes coding for type IV pili scattered in the genome. The type IV pili facilitate the attachment and invasion of prey cells. Extrachromosomal genetic elements such as plasmids were reported to be absent in the genomes of *B. bacteriovorus* HD100 except for a single copy of novel insertion sequence (IS) element ISBba77 and unknown *Bdellovibrio* prophage. Another notable feature that was reported about *B. bacteriovorus* HD100 genome is the absence of recent horizontal gene transfer (HGT) evident with the consistent average GC content. The presence of regions with unusual high GC content in the bacteria is usually an indication of lateral gene transfer events (Hayek, 2013). However, four regions coding for ribosomal lipopolysaccharide (LPS) synthesis, prophage and restriction modifications were observed to deviate from the average GC content in *B. bacteriovorus* HD100 are high in AT rich, and these regions have been reported to be generally higher in AT in any bacteria.

Table 5. 1. General features of the *B. bacteriovorus* HD100 genome (European Molecular Biology Laboratory accession no. BX842601). (Rendulic *et al*., 2004)

Species	*B. bacteriovorus*
Strain	HD100 (Deutsche Sammlung Mikroorganismen DSM50701)
Size	3,782,950 bp
GC content	50.7%
GC in coding areas	50.4%
Predicted number of ORFs	3584
Coding sequences (CDS)	1995
similar to known proteins	
Conserved hypothetical proteins	382
Hypothetical proteins	1207
Coding potential	93%
Average CDS length	982 bp
rRNA operons	2
tRNA genes	36
Mobile genetic elements	1 IS element
	4 transposases
Four regions of deviating GC content	LPS synthesis
	Prophage insertion in $tRNA^{met}$
	Ribosomal gene cluster
	Restriction modification system
CDS coding for hydrolytic enzymes	150 proteases/peptidases
	20 DNases
	9 RNases
	10 glycanases
	15 lipases
	89 other

The absence of recent horizontal gene transfer in *B. bacteriovorus* HD100 despite easy contact with prey genetic materials which can easily be incorporated into *B. bacteriovorus* genome raised a question whether it is resistant to foreign gene uptake.

The report that *Bdellovibrio* spp. are resistant to foreign DNA took a new twist when Gophna *et al*. (2006) reported that ancient lateral gene transfer has occurred in *B. bacteriovorus* HD100. The group first observed that *B. bacteriovorus* shared fewer genes with three δ-proteobacterial genomes including *Desulfotalea psychrophila*, *Desulfovibrio vulgaris* and *Geobacter sulfurreducens* when genome comparisons of the four genomes was carried out using the lineage-specific, group-specific and species-specific (coding) open reading frame (ORF) analysis. Furthermore, the aerobic pathways observed in *B. bacteriovorus* was suggested to be as a result of lateral gene transfer since the other three δ-proteobacteria are anaerobic chemolithotrophic sulfate reducers. Second evidence suggesting horizontal gene transfer in *B.*

bacteriovorus is the branching of *B. bacteriovorus* with *Leptospira interrogans* (a spirochete and non- δ-proteobacteria) in a discordance-weighted genome tree. Gophna *et al*. (2006) concluded that *B. bacteriovorus* has some coding genes (mostly genes involved in transport and hydrolysis) that have been acquired long ago and ameliorated into its genome, However, they further stated that these lateral gene transfers is not enough to cause genetic instability.

In 2011, Pan *et al*. reported for the first time that *B. bacteriovorus* has recently acquired AT-rich genes from Gram-negative bacteria other than the group δ-proteobacteria via horizontal gene transfer. In addition, they indicated that Cell-wall-surface-anchor-family proteins play a role in prey-predator interaction during the time of recognition, anchor and prey invasion.

B. bacteriovorus is regarded as obligate predator of other Gram-negative bacteria. However, the host independent strains that are capable of growing on nutrient rich media have been derived in the laboratory. The existence of host independent phenotypes of *B. bacteriovorus* in natural environment was reported by Hobley *et al*., (2012). The group isolated a strain of *B. bacteriovorus* designated Tiberius from River Tiber which is known to be rich in different bacteria and organic pollutants. The *B. bacteriovorus* Tiberius was able to grow simultaneously both as host dependent and independent phenotypes.

The genome of *B. bacteriovorus* Tiberius was observed to show significant conservation with *B. bacteriovorus* HD100 despite being isolated from different environmental habitats; aquatic and soil environment respectively. Hobley and co-workers further discovered that *B. bacteriovorus* Tiberius possess more incomplete phages and insertion sequences than the HD100 strain. Furthermore, approximately 91 – 92 % of genes acquired by ancient lateral gene transfer in *B. bacteriovorus* HD100 as reported by Gophna *et al*., (2006) was found in *B. bacteriovorus* Tiberius, and 3 out of 35 “recently acquired” reported by Pan *et al*. (2011) was found in the Tiberius strain. The genome comparison between the Tiberius strain and the HD100 strain supported the earlier report of Gophna et al. (2006) and Pan et al. (2011) that genome ancient and recent lateral gene transfer has contributed to the predatory evolution of *B. bacteriovorus*. Though the recent lateral gene transfer is minimal the strain Tiberius compared to the strain HD100. They also reported that

amelioration may have prevented the discovery of genes transferred horizontally. Other major finding of Hobley *et al*. (2012) is that genes transferred horizontally in *B. bacteriovorus* Tiberius are from the bacteria found in the aquatic-marine niche of River Tiber than from the sewage polluted part. On the contrary, in strain HD100, “the horizontally transferred” genes encoded product with BLAST top hits from bacteria associated with terrestrial habitats and plant.

The *Bd0108* region of the host interaction (locus) implicated in the conversion of *B. bacteriovorus* HD100 from the predatory form to the prey-independent phenotype (that is capable growing on nutrient-rich media) is the same with its homologue in *B. bacteriovorus* Tiberius (*bdt0101*) except that there are three single nucleotide polymorphism (SNPs) in the amino acid sequence of the strain Tiberius: $V^{HD100}31A^{tiberius}$, A86T and the less conservative substitution G97S. The group further speculated that the amino acid difference stated above might be responsible for the ability of *B. bacteriovorus* Tiberius to grow as prey-independent strain.

In this study, genomes of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 were sequenced. Comparative genomics tools were used to examine their closeness and relationship with other reported genomes retrieved from the NCBI database.

5.2. MATERIALS AND METHODS

5.2.1. Extraction of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 genomic DNA.

The method for the genomic DNA extraction and molecular confirmation of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 based on PCR amplification and sequencing of the 16S rRNA gene were done as described in Chapter 3.0, Section 3.1.3.

5.2.2. Complete whole genome sequencing of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315

The genomic DNA of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 were sequenced at myGenomics, LLC (Alpharetta, Georgia, USA) using Illumina® Nextseq Pair-End sequencing technology. The pair-end sequencing technique involves sequencing both ends of DNA fragments in a library. The procedure for illumina sequencing technology include fragmentation of genomic DNA to generate <800 bp fragments. The fragments generated are blunt ended and phosphorylated, and a single 'A' nucleotide is added to the 3' ends of the fragments before the addition of adaptors and additional motifs such as sequencing binding sites, indices, and regions complementary to oligonucleotides present in a flow cell to the end of the DNA fragments (Illumina, 2009). Each fragment molecule is then isothermally amplified through bridge amplification in a flow cell in a process called clustering. The sequencing then follows with the addition of fluorescently-tagged nucleotides in a process called sequencing - by- Synthesis to generate millions of reads (Illumina, 2010). The workflow for Pair-end sequencing technology is shown below. (Figure 5. 1). Based on the protocol obtained from myGenomics LLC Company, the sequencing of the *Bdellovibrio* strains is described as follows: The gDNA was subjected to optical density measurements in NanoDrop and Qubit (ThermoFisher Scientific, Waltham, MA, USA), DNA migration in agarose gel electrophoresis to confirm the purity and concentration prior to fragmentation in Bioruptor (Diagenode, Inc., Denville, NJ USA). Fragmented gDNA was tested for size distribution and concentration using a 2200 Tapestation (Agilent Technologies, Inc., Santa Clara, CA USA), and subjected to Illumina library preparation using Beckman SPRI-TE automated liquid handler and library prep reagents (Beckman Coulter, CA USA). The resulting library was tested for size distribution and concentration by 2200, NanoDrop and Qubit. The libraries were then loaded for Illumina NextSeq sequencing according to the standard operation. Paired-end 75 nucleotide (nt) reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK).

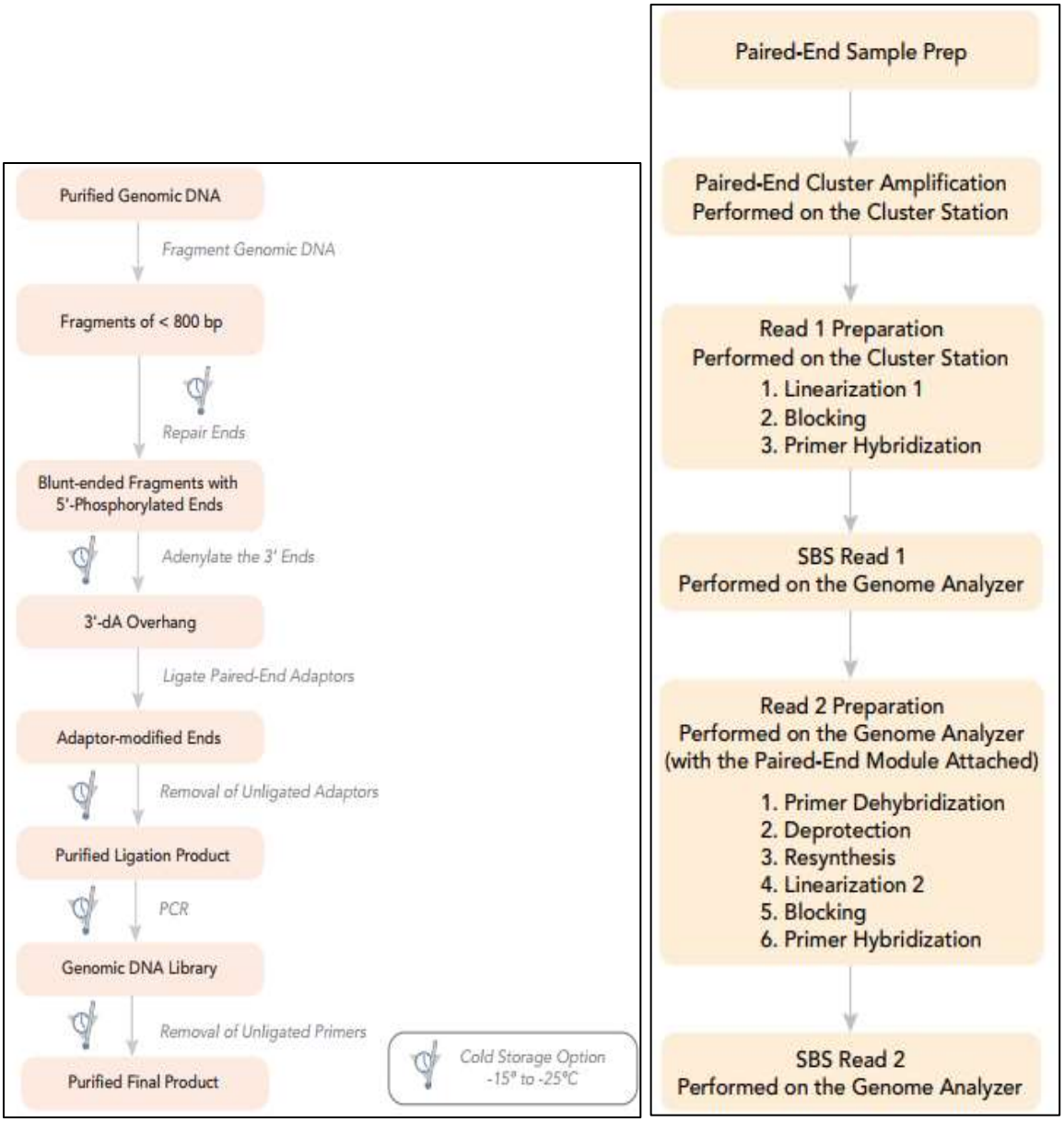


Figure 5. 1. The workflow for sample preparation and Paired-End sequencing protocol (Illumina Inc.)

5.2.3. Cleaning of Reads obtained from illumina sequencing

The pair-end reads obtained after illumina sequencing was received from myGenomics, LLC Company in a .fastq format. The pair-end reads were trimmed and cleaned using a window adaptive trimming tool for FASTQ files known as Sickle, in order to remove adapters and reads of low quality that can affect the genome assembly and other bioinformatics downstream analyses.

Sickle is a tool that uses sliding windows along with quality and length thresholds to determine when quality is sufficiently low to trim the 3'-end of reads and also determines when the quality is sufficiently high enough to trim the 5'-end of reads (Joshi and Fass, 2011).

5.2.4. Genome sequence assembly and generation of Scaffolds for *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315

The trimmed pair-end reads were assembled de-novo using SPAdes assembler version 3.10.0 (Bankevich *et al*., 2012). The SPAdes uses four steps to assemble reads including (i) assembly graph construction using the multisized de Bruijn graph to detect and remove chimeric reads; (ii) *k*-bimer adjustment to derive accurate distance estimates between *k-mers* in the genome (edges in the assembly graph); (iii) construction of paired assembly graph; (iv) Construction of contigs and the mapping of reads to contigs by backtracking graph simplifications.

The contigs obtained from the assembly of reads using the SPades software were arranged into scaffold using Multi-Draft based Scaffolder; MeDuSa v1.4 (Bosi *et al*., 2015). Genome scaffolding involves the process of ordering and orienting contigs. MeDuSa v1.4 orders and orientates contigs using complete close reference genomes. Bosi *et al*. (2015) highlighted the advantages of using MeDuSa scaffolder as follows: (i) formalizes the scaffolding problem by means of a combinatorial optimization formulation on graphs and implements an efficient constant factor approximation algorithm to solve it; (ii) allows for multiple reference genomes to be used during scaffolding; (iii) does not require prior knowledge on the evolutionary relationships (i.e. a phylogenetic tree) among the reference set of organisms and (iv) can handle both draft and complete reference genomes. In this study, using the default settings, the scaffolds for the Bdellovibrio strains contigs were generated using the following reference genomes: complete genomes of four *B. bacteriovorus* strains (*B. bacteriovorus* W; NZ_CP002190, *B. bacteriovorus* HD100; NC_005363; *B. bacteriovorus* str. Tiberius; NC_019567, *B. bacteriovorus* 109J; NZ_CP007656) and three draft genomes (*B. bacteriovorus* R0; LUKE00000000, *B. bacteriovorus* EC13; LUKD00000000; *B. bacteriovorus* BER2 LUKF00000000) retrieved from the NCBI database.

The resulting scaffolds were then improved using Iterative Mapping and Assembly for Gap Elimination; IMAGE (Tsai *et al*., 2010). IMAGE software improves the quality of draft genome by using local assemblies of reads from gap regions. From the large numbers of sequences obtained from the illumina sequencing, IMAGE identifies reads that correspond to gaps or questionable regions, reassemble them locally before incorporating them back into the final assembly.

5.2.5. Genome annotations of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315

The genome annotation of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315 were automatically annotated using NCBI Prokaryotic Genome Annotation Pipeline; PGAP (Tatusova *et al*., 2016). The PGAP pipeline execute genome annotation based on the following: (i) the pipeline uses a pan-genome approach to protein annotation with pan-genome proteins defined for a specific clade; (ii) the pipeline incorporates additional specialized search tools to identify novel nonprotein-coding functional elements, including CRISPR regions; (iii) identify protein-coding genes the pipeline using a two-pass approach designed to detect frameshifted genes and pseudogenes; (iv) uses GeneMarkS+, that integrates extrinsic information (alignment based protein predictions, predicted RNA genes, etc.) with intrinsic information on genome-specific sequence patterns of protein-coding regions. The assembled genomes were also submitted to RAST (Rapid Annotation using Subsystem Technology) server for automated annotation. The RAST achieve genome annotation by assigning genes to different manually curated subsystems and finally use the information for an online metabolic reconstruction annotation (Aziz *et al*., 2008).

5.2.6. Identification of prophages sequences, Genomic Islands and predation-enhancing factors present in the assembled and annotated genomes of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315

The presence of prophage sequences and genomic islands in the genomes of bacteria contributes to diversity in bacterial groups. Moreover, acquisition of prophages or genomic islands can confer on bacteria different adaptive features such as resistance to antibiotics, pathogenicity, and ability to colonize and survive in different environments. Genomic islands are probably acquired by bacteria via Horizontal Gene Transfer and have been implicated in genome evolution (Aminov, 2011). Identification of prophage sequences and genomic islands in *Bdellovibrio* spp. SKB1291214 and SSB218315 was achieved using online software package known as PHASTER (PHAge Search Tool – Enhanced Release) and IslandViewer 4 respectively. PHASTER software identifies prophage sequences by BLAST searching input genome sequence in GenBank or FASTA format against prophage/phage sequences in the National Center for Biotechnology Information (NCBI) phage database and the prophage database. IslandViewer 4 webserver incorporates four genomic island prediction methods including (i) IslandPick (Langille *et al*., 2008) which uses a comparative genomics-based approach, identifying unique regions by comparing a user-specified genome against closely related genomes; (ii) IslandPath-DIMOB (Hsiao *et al*., 2003) which identify islands with dinucleotide bias and the presence of an associated mobility gene (integrases, transposases, etc.); (iii) SIGI-HMM (Waack *et al*., 2006) which identifies codon usage bias with a hidden Markov model approach and (iv) Islander which predicts genomic island based on mechanistic consequences of their typical site-specific integration into tRNA/tmRNA genes.

The GenBank formats of the genome sequences of *Bdellovibrio* spp. SKB1291214 and SSB218315 were submitted to the webserver of PHASTER (http://phaster.ca/) and IslandViewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/upload/) to predict the presence of prophages and genomic islands using default settings following instruction as recommended on the online page. *B. bacteriovorus* HD100 was used as reference strain for the prediction of genomic islands in *Bdellovibrio* sp. SKB1291214. Furthermore, different factors that aid the successful predation of *Bdellovibrio* spp. SKB1291214 and SSB218315 was identified by carrying out Basic Local

Alignment Search Tool (BLAST) analysis of the genomes in the Virulence Factor Database (VFDB) using lower expect value of 0.01 to increase the stringency of the predictions (Chen *et al*., 2005). The VFDB was originally designed to provide information on the virulence factors (VFs) from bacterial pathogens of medical importance. However, it can also provide information on the VFs or predation-enhancing factors that *Bdellovibrio* can use for survival during predation. Therefore, the predicted VFs obtained from VFDB were further manually searched and analyzed from *Bdellovibrio* strains genomes.

5.2.7. Whole-genome based species identification of *Bdellovibrio* spp. SKB1291214 and SSB218315

The ANI/AAI-Matrix Genome-based distance matrix calculator was used to determine the genetic relatedness of nine *Bdellovibrio* strains by calculating the percentage average amino acid identity (AAI) of their genome. Whole genome sequencing has paved way for microbial taxonomy based on the evolutionary information contained in the genome sequences, such as the Karlin genomic signatures, Average Amino Acid Identity (AAI), supertrees, and in silico Genome-to-Genome Distance Hybridization; GGDH (Konstantinidis & Stackebrandt, 2013). The determination of average nucleotide identity (ANI) and AAI have been shown to be useful in distinguishing or delineating species of prokaryotes (Konstantinidis & Tiedje (2005). AAI is based on using BLAST algorithm to carry out whole-genome pairwise sequence comparisons in order to determine conserved protein-coding genes between a pair of genomes. Microbial strains that share >95% AAI and ANI, >95% identity based on multiple alignment genes are usually considered to be of the same species (Thompson *et al*., 2013).

In addition to the two sequenced whole genomes (*Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315), complete genomes of four *Bdellovibrio bacteriovorus* strains (*B. bacteriovorus* W; NZ_CP002190, *B. bacteriovorus* HD100; NC_005363; *B. bacteriovorus* str. Tiberius; NC_019567, *B. bacteriovorus* 109J; NZ_CP007656) and three draft genomes (*B. bacteriovorus* R0; LUKE00000000, *B. bacteriovorus* EC13; LUKD00000000; *B. bacteriovorus* BER2 LUKF00000000) were retrieved from the NCBI database and used to calculate the AAI. A

heatmap of the average amino acid identity matrix clustering was generated in R package plots using the heatmap.2 function.

5.2.8. Phylogenetic Tree Construction and Estimation of Pairwise Evolutionary Divergence between 16S rRNA gene sequences.

The 16S rRNA gene sequences were retrieved from the genomes of the *Bdellovibrio* spp used to calculate the AAI and used to construct a phylogenetic tree and estimate Pairwise Evolutionary Divergence. This analysis provides a comparison and confirmation of result obtained from the AAI calculation. The 16S rRNA gene sequences were aligned using Muscle alignment tool with default parameters and a phylogenetic tree was constructed using the Maximum Likelihood method based on the Kimura 2- parameter model. Bootstrap values were calculated to test the robustness of interior node support and were obtained by conducting 1000 pseudoreplicates using MEGA© 6.0 software (Tamura *et al*., 2013). Pairwise Evolutionary Divergence (distance) was conducted in MEGA© 6.0 software using Kimura 2- parameter model with 1000 bootstrap replications.

5.2.9. Determination of genome-wide orthologous gene clusters

The genome-wide orthologous gene clusters analysis between the study genomes and four complete genomes retrieved from NCBI (*B. bacteriovorus* W; NZ_CP002190, *B. bacteriovorus* HD100; NC_005363; *B. bacteriovorus* str. Tiberius; NC_019567, *B. bacteriovorus* 109J; NZ_CP007656) was performed using Orthovenn, with the E-value and inflation value set as 1e-5 and 1.5 respectively (Wang *et al*., 2015). Orthologous genes originate from common ancestors by speciation or vertical descent (Koonin, 2005). Orthovenn provides an efficient and interactive graphics tool to provide a Venn diagram view of the genome-wide comparison of orthologous clusters based on the protein sequence data selected from up to six species. It uses the popular heuristic approach named OrthoMCL (Li *et al*., 2003) to identify ortholog groups utilizing UBLAST (v7.0.1090) (Edgar, 2010) to do the all-against-all similarity search instead of BLASTP. UBLAST has been described to be ~350× faster than BLASTP and achieves very similar results for ortholog searches (Moreno-Hagelsieb and Hudy-Yuffa, 2014). A multithreaded C application known as

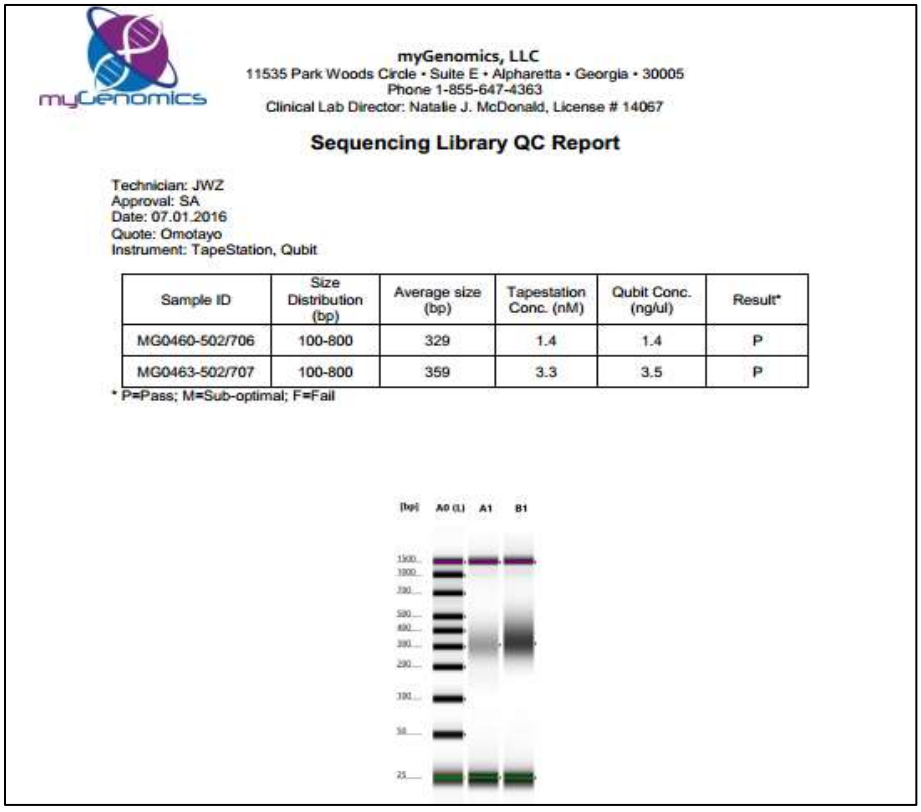
orthAgogue (v1.0.3) is then used to identify putative orthology and inparalogy relations (Ekseth *et al*., 2014). Finally, BLASTP analysis is used to determine the putative function of each ortholog.

5.2.10. Whole genome alignment and visualization of Synteny among *Bdellovibrio* spp.

Genome alignment and Visualization of synteny among selected *Bdellovibrio* spp was done using ProgressiveMAUVE, a part of Mauve v2.3.1 (Darling *et al*., 2004) genome alignment package. Mauve is a multiple sequence alignment package that identifies and aligns regions of local collinearity called locally collinear blocks (LCBs) containing sequence elements conserved among all the genomes being aligned. The *Bdellovibrio* spp. considered for the alignment include *Bdellovibrio* sp. SKB1291214, *B. bacteriovorus* SSB218315, *B. bacteriovorus* W; NZ_CP002190, *B. bacteriovorus* HD100; NC_005363; *B. bacteriovorus* str. Tiberius; NC_019567, *B. bacteriovorus* 109J; NZ_CP007656), *B. bacteriovorus* R0; LUKE00000000, *B. bacteriovorus* EC13; LUKD00000000 and *B. bacteriovorus* BER2 LUKF00000000. Circular genome alignment of the nine *Bdellovibrio* spp was done using BLAST Ring Image Generator (BRIG) v0.95 (Alikhan *et al*., 2011). Finally, circular. Circular genome diagrams of *Bdellovibrio* spp SKB1291214 and SSB218315 that display different features of the genomes was constructed using DNAplotter of the Wellcome Trust Sanger Institute (Carver *et al*., 2019).

5.3. Complete whole genome sequencing of *Bdellovibrio* sp. SKB1291214 and *B. bacteriovorus* SSB218315

The reports below (Figure 5. 2) was obtained from the sequencing company, myGenomics LLC. It shows the quality of the genomic DNA after fragmentation using Bioruptor NGS. For pair-end Illumina sequencing, the fragments generated should be <800 bp. From the report the size distribution after fragmentation was observed to be between 100 – 800 bp with an average size of 329 bp and 359 bp for *Bdellovibrio* spp. SKB1291214 and SSB218315 respectively.



myGenomics, LLC
11535 Park Woods Circle • Suite E • Alpharetta • Georgia • 30005
Phone 1-855-647-4363
Clinical Lab Director: Natalie J. McDonald, License # 14067

Sequencing Library QC Report

Technician: JWZ
Approval: SA
Date: 07.01.2016
Quote: Omotayo
Instrument: TapeStation, Qubit

Sample ID	Size Distribution (bp)	Average size (bp)	Tapestation Conc. (nM)	Qubit Conc. (ng/ul)	Result*
MG0460-502/706	100-800	329	1.4	1.4	P
MG0463-502/707	100-800	359	3.3	3.5	P

* P=Pass; M=Sub-optimal; F=Fail

Figure 5. 2. Report obtained from myGenomics LLC. The report shows the quality (size and concentration) of genomic DNA after fragmentation using Bioruptor NGS in preparation for Illumina® Nextseq pair-end sequencing technology and gel image showing the size distribution of the fragmented genomic DNA using a 2200 Tapestation. The myGenomics LLC certified the genomic DNA suitable for Illumina® Nextseq pair-end sequencing technology.

5.4. Genomic features of *Bdellovibrio* spp. strains SKB1291214 and SSB218315.

The genomic features of *B. bacteriovorus* strain SSB218315 and *Bdellovibrio* sp. strain SKB1291214 are summarized in Table 5.1. The complete circular genome of strain SSB218315 yielded a genome size of 3,769,537 bp with GC content of 50.5 %. The PGAP predicted 3534 protein-coding sequences (CDS) and 41 RNAs, including 34 tRNAs, 3 rRNAs, and 4 non-coding (nc) RNAs. For *Bdellovibrio* sp. strain SKB1291214, the draft genome size is 3,724,490 bp consisting of 20 scaffolds and GC content of 44.8 %. The circular genomic map of *Bdellovibrio* spp. SKB1291214 and SSB218315 are shown in Figure 5. 3. The PGAP predicted 3552 CDS and 40 RNAs - 33 tRNAs, 3 rRNAs, and 4 ncRNAs. The genomic features of *Bdellovibrio* spp. SKB1291214 and SSB218315 obtained from the online prokaryotic genome annotation service RAST is presented. The predicted genes are distributed into different subsystems categories based on their functional roles by the RAST system (Figure 5. 4 and 5. 5).

Table 5. 2. The genomic features of *Bdellovibrio* spp. SSB218315 and SKB1291214.

Genome Information	***B. bacteriovorus* SSB218315**	***Bdellovibrio* sp. SKB1291214**
Chromosome size	3,769,537 bp	3,724,490 bp
Number of contigs	1	20
N_{50}	-	199,513
GC Content	50.5 %	44.80 %
Total RNA	41	40
Complete rRNAs	3	3
tRNAs	34	33
Non-coding RNAs	4	4
Total genes	3,620	3,676
Total CDS	3,579	3,636
Coding CDS	3534	3552
Phage	1(incomplete)	1(incomplete)
Genomic Island (Integrated method)	23	132

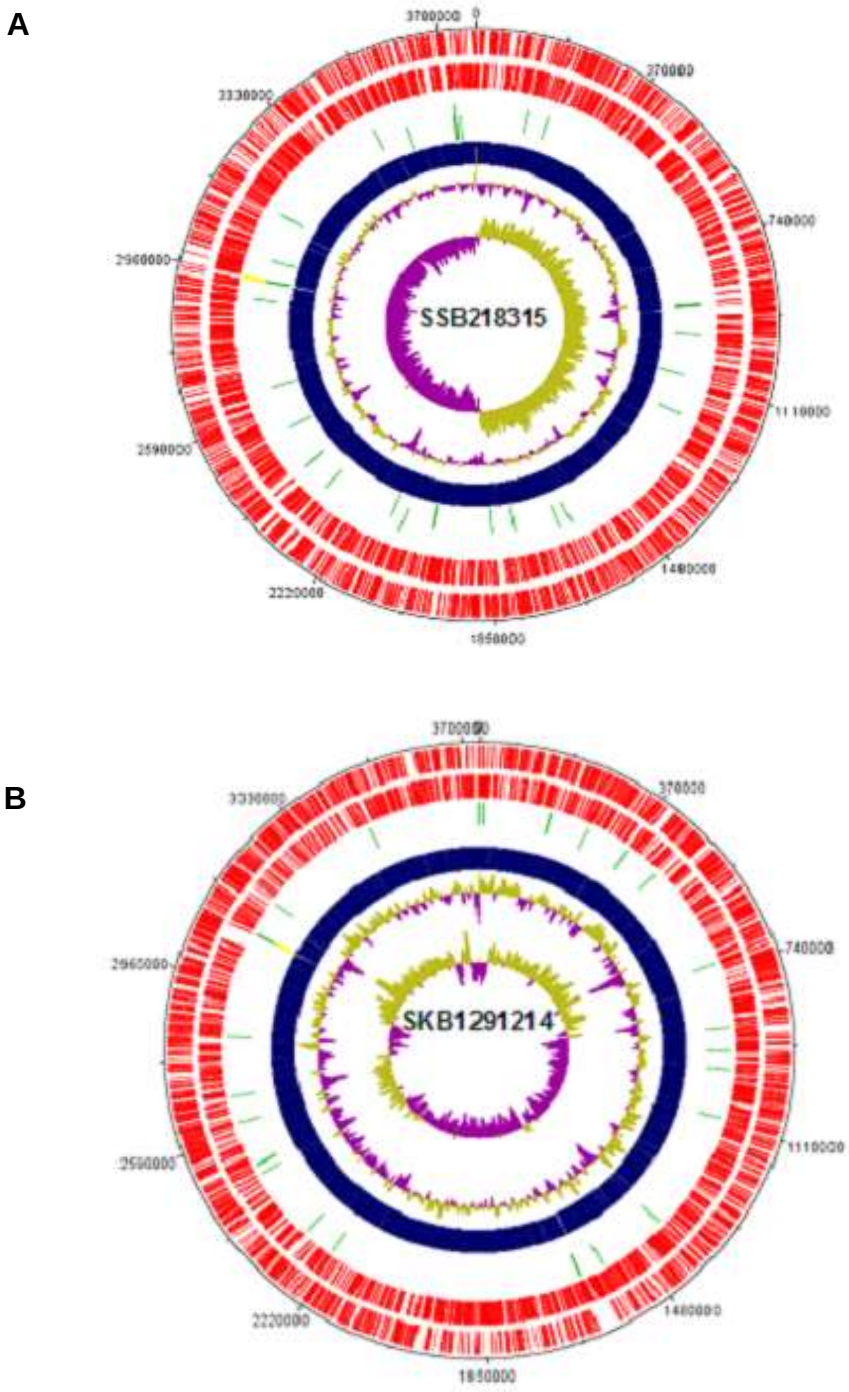


Figure 5. 3. The circular genomic map of *Bdellovibrio* spp. strains (A). SSB218315 and (B). SKB1291214 (in this study) drawn using DNAplotter software. From outside to center: ORFs on the positive strand (Red); ORFs on the negative strand (Red); tRNA (Green); regions of homology (dark blue); rRNA (yellow)% GC content; GC skew.

Size	3,769,537
GC Content	50.5
L50	1
Number of Contigs (with PEGs)	1
Number of Subsystems	358
Number of Coding Sequences	3592
Number of RNAs	39

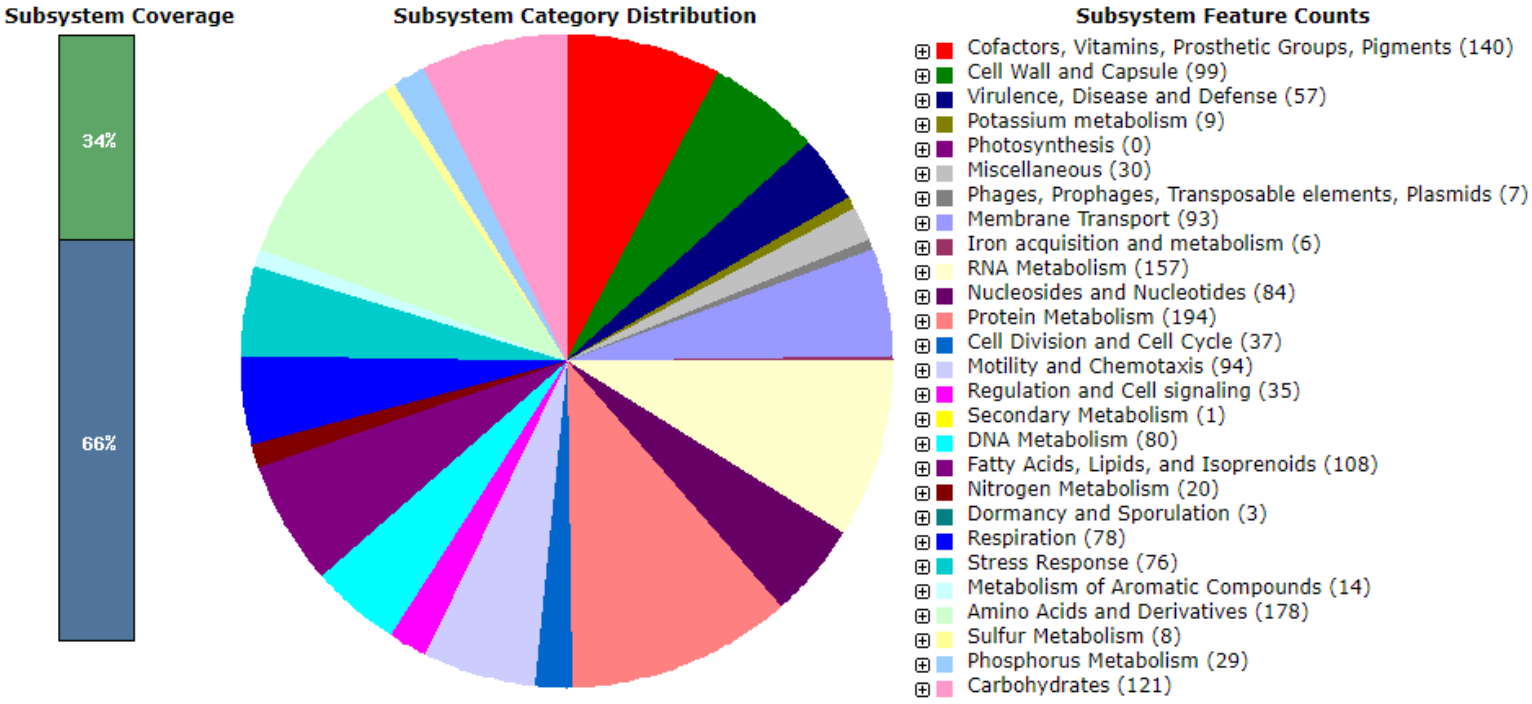


Figure 5. 4. Graphical image showing the genome annotation of *B. bacteriovorus* SSB218315 genome in RAST. It shows the distribution of the genes into different subsystem categories based on their functional roles.

Size	3,730,460
GC Content	44.8
N50	199513
L50	7
Number of Contigs (with PEGs)	20
Number of Subsystems	349
Number of Coding Sequences	3664
Number of RNAs	36

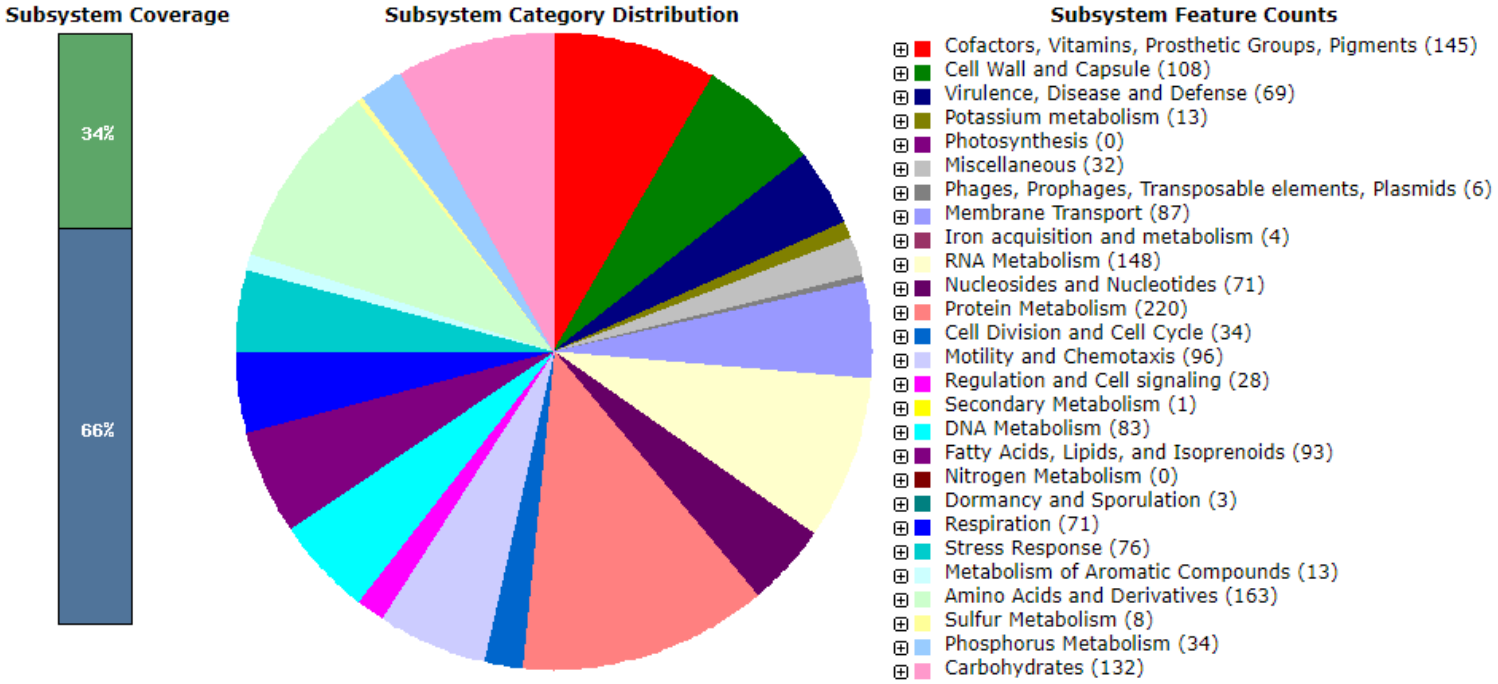


Figure 5. 5. Graphical image showing the genome annotation of *Bdellovibrio* sp. SKB1291214 genome in RAST. It shows the distribution of the genes into different subsystem categories based on their functional roles

The PHAge Search tool (PHASTER) identified one incomplete prophage in each sequenced genome. In *B. bacteriovorus* SSB218315 genome, the incomplete prophage has a total of 12 proteins (CDS), percentage GC content of 50.61 % and a size of 14.6 kb (Table 5.1, Figure 5. 6.) Meanwhile, prophage identified in *Bdellovibrio* sp. SKB1291214 has region length of 11.8 kb with 13 CDS and percentage GC content of 42.2 % (Table 5. 1, Figure 5. 7).

In *B. bacteriovorus* SSB218315, the genomic islands were only predicted by the SIGI-HMM method incorporated into the IslandViewer 4 program. The total number of genomic islands predicted were 23. The predicted genomic islands include 16 hypothetical proteins, peptidase S74, TetR family transcriptional regulator, patatin, transcriptional regulator, septation protein (spoVG), lipid carrier--UDP-N-acetylgalactosaminyltransferase and UDP-N-acetylenolpyruvoylglucosamine reductase (Appendix 3). In addition, the IslandViewer also identified a region coding for elongation factor Tu (*tuf* gene) and categorized it as a homolog of resistance gene in the IslandViewer 4 software. However, in *Bdellovibrio* sp. SKB1291214, IslandViewer 4 predicted a total of 132 genomic islands. The SIGI-HMM predicted 18 genomic islands including 11 hypothetical proteins, aldehyde-activating protein, an integrase, AlpA family phage regulatory protein, terminase small subunit, and XRE family transcriptional regulator. Meanwhile, the Islandpath-Dimob program predicted 114 genomic islands which include some flagella associated proteins, integrase, insertase, exinuclease, ABC and MFS transporters, chemotaxis associated genes, endopeptidase, chromosome partitioning protein, HAD phosphatase, ribonuclease P protein component, pyridoxal phosphate biosynthetic protein, survival protein SurA and other proteins (Appendix 4). The maps showing the distribution of the genomic islands in the genome of *Bdellovibrio* spp. SSB218315 and SKB1291214 are shown in Figure 5. 8 and Figure 5. 9 respectively. It is also important to mention that no pathogen associated genes were found in the genomes of the two *Bdellovibrio* spp. This same result was observed when the sequenced genomes of the two Bdellovibrio spp. were submitted to the Pathogenic Island Database (PAIDB) for identification of possible presence of pathogenic islands. Furthermore, the PathogenFinder, a software program developed by Center for Genomic Epidemiology predicted the two *Bdellovibro* spp. as non human pathogens (Appendix 1 and 2).

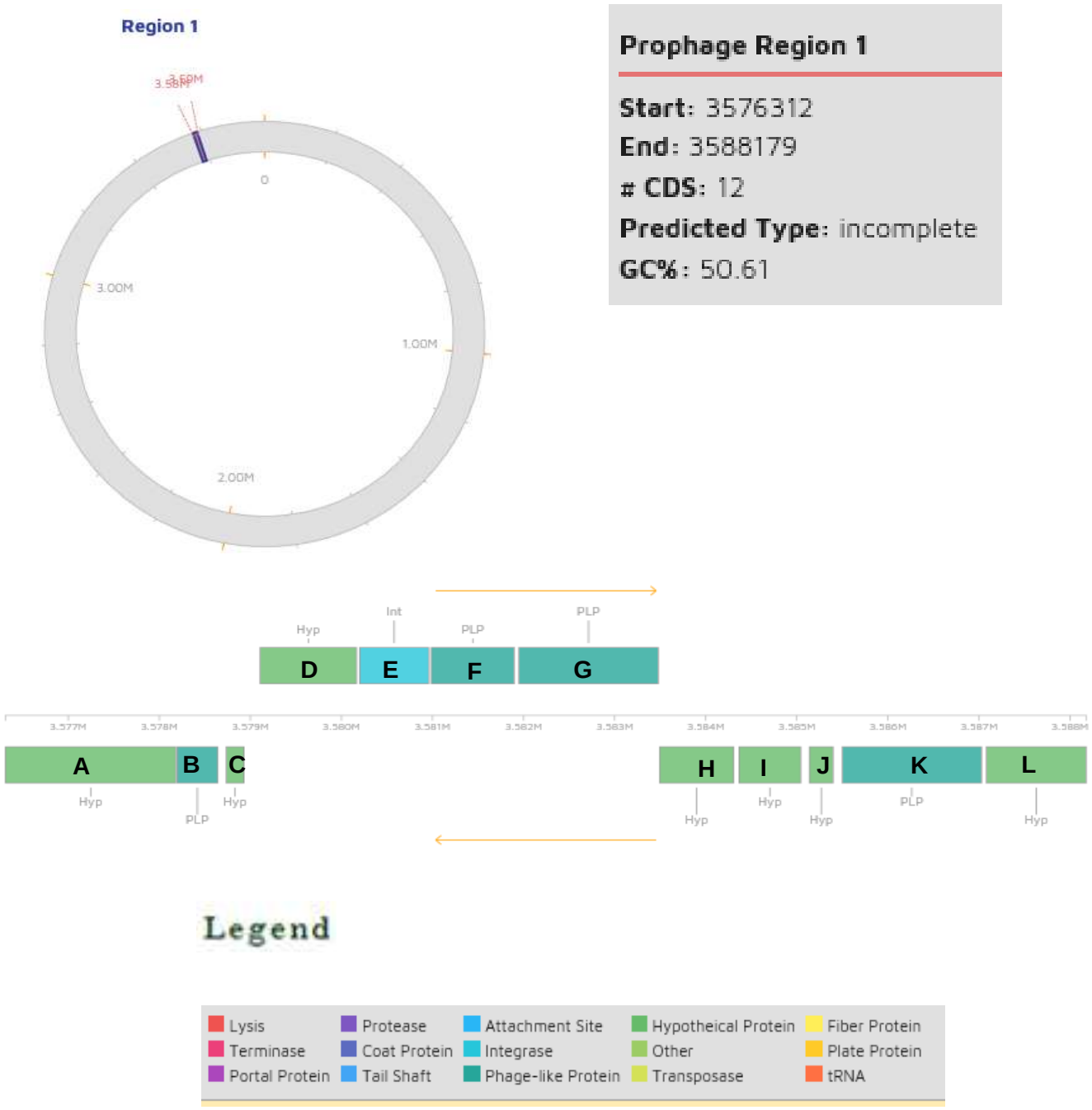


Figure 5. 6. Map showing the position of the incomplete prophage identified in the genome of *B. bacteriovorus* SSB218315. The BLASTP analysis of each protein in the region in the NCBI is as follows: **A.** DNA primase; **B.** Glutamyl-tRNA amidotransferase; **C.** 30S ribosomal protein S21; **D.** Hypothetical protein (domain hit: pfam15887-Peptidase_Mx); **E.** Hypothetical or membrane protein (domain hit: pfam03741, COG0861-TerC); **F.** tRNA adenosine deaminase TadA; **G.** ATP-dependent helicase; **H.** Glutamine amidotransferase; **I.** DNA-binding response regulator; **J.** Translation initiation factor, **K.** Sporulation (SpoVR) protein, **L.** Stress response protein.

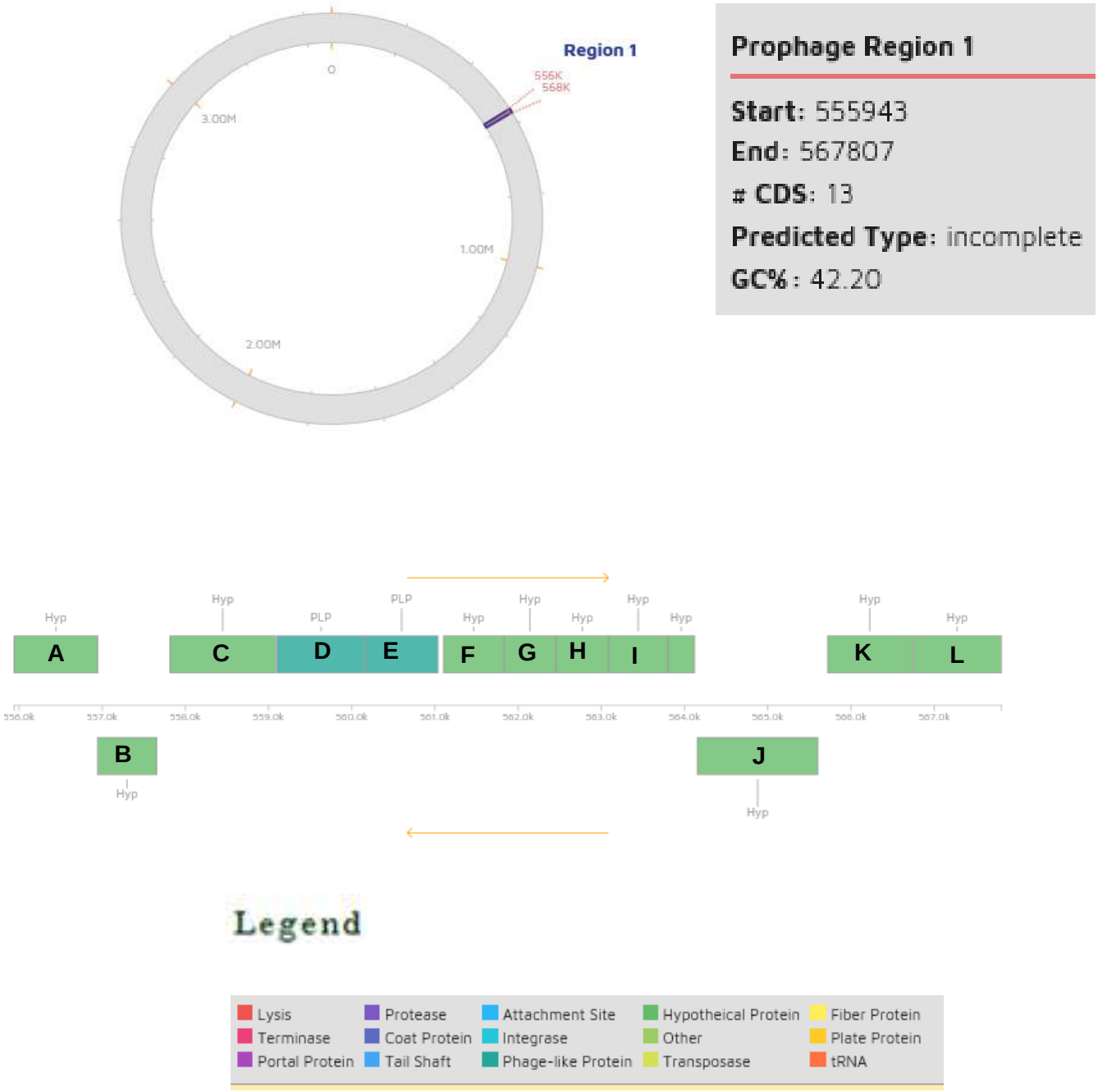


Figure 5. 7**.** Map showing the position of the incomplete prophage identified in the genome of *Bdellovibrio* sp. SKB1291214. The BLASTP analysis of each protein in the region in the NCBI is as follows: **A.** CDP-glucose 4,6-dehydratase; **B.** family 2 Glycosyl transferase; **C.** Hypothetical or O-Antigen ligase (domain hit: pfam04932, COG3307); **D.** dTDP-glucose 4,6-dehydratase; **E.** Glucose-1-phosphate thymidylyltransferase ; **F.** Glycosyl transferase family 2; **G.** Phosphodiesterase; **H.** HAD family phosphatase; **I.** Glycosyl transferase family 2; **J.** Hypothetical protein, **K.** Hypothetical (domain hit: cd13128-MATE_Wzx_like, COG2244-RfbX), **L.** Hypothetical protein (domain hit: pfam07507-WavE), **M.** Acyltransferase.

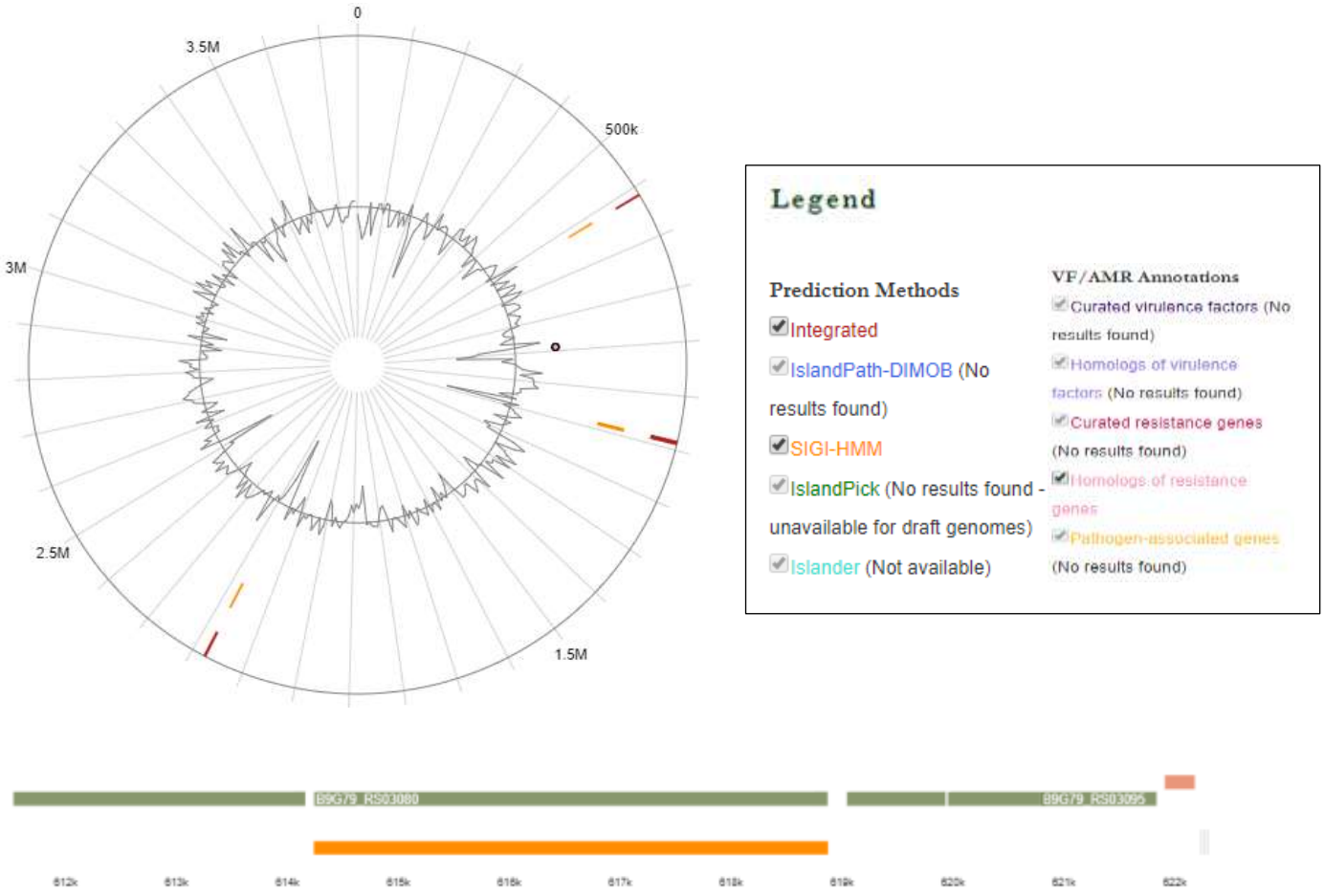


Figure 5. 8. The genomic map showing the distribution of genomic islands in the genome of *B. bacteriovorus* SS218315 identified using software IslandViewer 4.

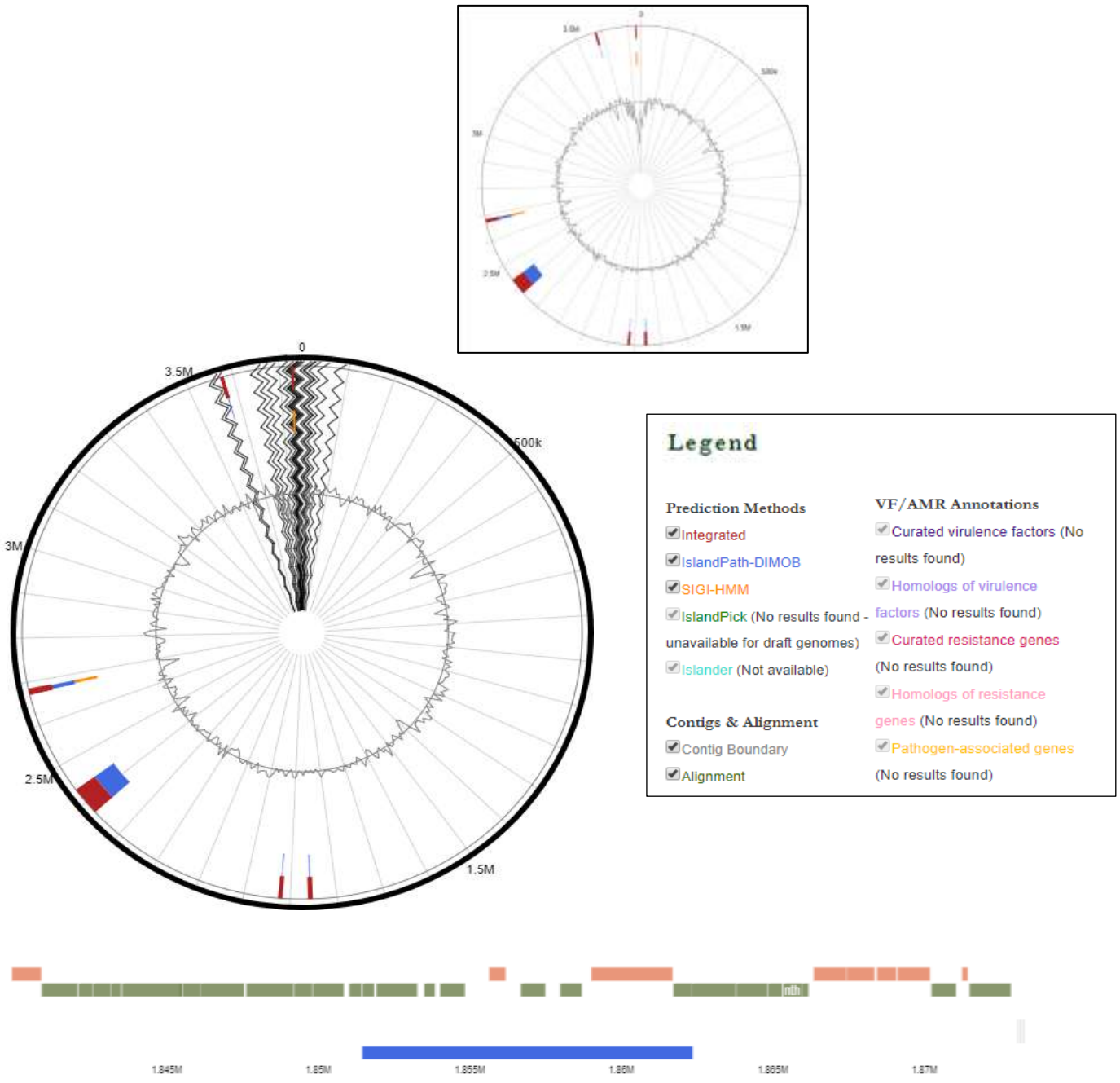


Figure 5. 9. The genomic map showing the distribution of genomic islands in the genome of *Bdellovibrio* sp. SKB1291214 identified using software IslandViewer 4. The thick black line around the genome indicate genome alignment with a reference genome *B. bacteriovorus* HD100 while the zig-zag black lines indicates contig boundary. Inset (genome map when the alignment line and contig boundary line is removed).

BLAST search analysis in the VFDB and manual curation revealed the presence of different virulence factors that could aid the survival of the *Bdellovibrio* strains SKB1291214 and SSB218315 as predatory

bacteria (Table 5.2). Among the virulence or predation-enhancing factors are motility factors coding for flagella development and flagellar peptidoglycan-hydrolyzing activity (*flgJ*), and also chemotaxis factors. Some adherence factors that can facilitate the attachment of the *Bdellovibrio* strains to prey as well as twitching motility along the prey cells were also found in the genomes. The adherence factors include type IV pili system involving the *pil* genes, and heat shock proteins. Other virulence factors include genes coding for periplasmic catalase/(hydro)peroxidase, peptidases (endopeptidases, peptidase M14, and carboxypeptidase), proteases (metalloproteases and ATP-dependent *Clp* protease proteolytic subunit) and peptide methionine sulfoxide reductase *MsrA*/*MsrB*. Lipases, esterases, nucleases, siderophores, alpha-amylase and pullulanase were also found in the genomes. The virulence factors found in the genomes and observed to be similar to pathogenic factors that have been reported in pathogenic bacteria such as *Staphylococcus aureus* (Otto, 2014) and *Vibrio* spp. (Miyoshi, 2013) were RTX toxins, hemolysins and collagenases.

Table 5. 3. The various virulence or predation-enhancing factors found in the genomes of *B. bacteriovorus* SSB218315 and *Bdellovibrio* sp. SKB1291214 and their functions.

PREDATION FACTORS	FUNCTIONS
MOTILITY ASSOCIATED FACTORS:	Responsible for high motility of *Bdellovibrio* spp. during the attack phase
Flagellar biosynthesis proteins	Flagellar rod assembly and degradation of peptidoglycan
Flagellar protein, peptidoglycan hydrolase (*flgJ*)	Responsible for the motility of *Bdellovibrio* spp on solid surfaces or area of low water content such as biofilms, microbial mats or soil
Adventurous gliding motility protein and gliding motility ABC transporter (U,S,R, V, MgIA and T)	
TYPE IV PILUS ASSOCIATED FACTORS:	Essential for *Bdellovibrio* spp. attachment and invasion of prey cells
Type IV pilus biogenesis and assembly protein	This is essential for type IV fimbrae or pilus biogenesis
PilZ domain-containing protein	
CHEMOTAXIS ASSOCIATED FACTORS:	Mediates response of bacteria towards chemical signals
Chemotaxis protein methyltransferase (*cheR*), Chemotaxis response regulator protein-glutamate methyltransferase (*cheB*), Chemotaxis protein (*cheX*), Methyl accepting chemotaxis protein (*cheD*).	
Chemotaxis response regulator (*cheY*)	Transmits chemoreceptor signals to flagellar motor components
Protease and peptidase	Catalyze the cleavage of peptide bond in a protein or peptide by hydrolysis
Nuclease	Hydolytic degradation of nucleic acids
Lipase and Esterase	Hydrolysis of lipids and ester bonds
Catalase and Peroxidase	Stress proteins involved in the intracellular survival of *Bdellovibrio* spp.
Siderophores	High affinity Iron chelating compounds for iron acquisition
Alpha-amylase and pullulanase	Degradation of complex polysaccharides
Collagenase	For breakdown of peptides in collagen
Hemolysin	Act as oligomeric pore forming proteins involve in cell lysis. It can also cause lysis of red blodd cells
RTX toxins	Family of cytolysins and cytotoxins
Methionine sulfoxide reductase (*MsrA/MsrB*)	Implicated in adherence and repair of oxidized proteins for intracellular survival.

5.5. Phylogeny and Amino Acid Identity of *Bdellovibrio* spp. strains SKB1291214 and SSB218315.

Species delineation was done by examining the AAI among the *Bdellovibrio* strains. For strains to belong to the same species, they must have ANI and AAI ≥ 95 %, <10 Karlin genomic signature and > 70 % *in*

silico GGDH (Konstantinidis and Tiedje, 2007). The AAI among all the *Bdellovibrio* strains ranges between 64–99 %. The AAI between strain SKB1291214 and other strains was very low (64 – 68 %) while strain SSB218315 shared high AAI value of 95 % with *B. bacteriovorus* strains HD100, Tiberius and 109J (Figure 5. 10; Appendix 5 and 6). Furthermore, *B. bacteriovorus* EC13 and BER2 were also observed to share high AAI of 96 %. Furthermore, the level of relatedness among the *Bdellovibrio* strains was further analyzed by constructing a phylogenetic network and tree using 16S rRNA gene sequences retrieved from each genome of the *Bdellovibrio* strains with NeighbourNet algorithm of splitsTree4 (Figure 5. 11) and MEGA6 (Figure 5. 12) software respectively. The result of the phylogenetic network correlates with the result of the AAI which show the *Bdellovibrio* strains clustering based on the percentage AAI they shared. As observed in the phylogenetic analysis result in objective 1, the 16S rRNA sequence of strains SKB1291214 and SSB218315 showed 96 % similarity with a pairwise evolutionary distance of 0.043. The strain SKB1291214 shared 100 % identity with an uncultured *Bdellovibrio* sp. clone12 L 106 (a pairwise distance of 0.008) while strain SSB218315 shared 100 % identity with other culturable terrestrial *B. bacteriovorus* which include *B. bacteriovorus* strain HD100 (pairwise distance 0.001) and Tiberius (pairwise distance 0.004) (Table 5.3).

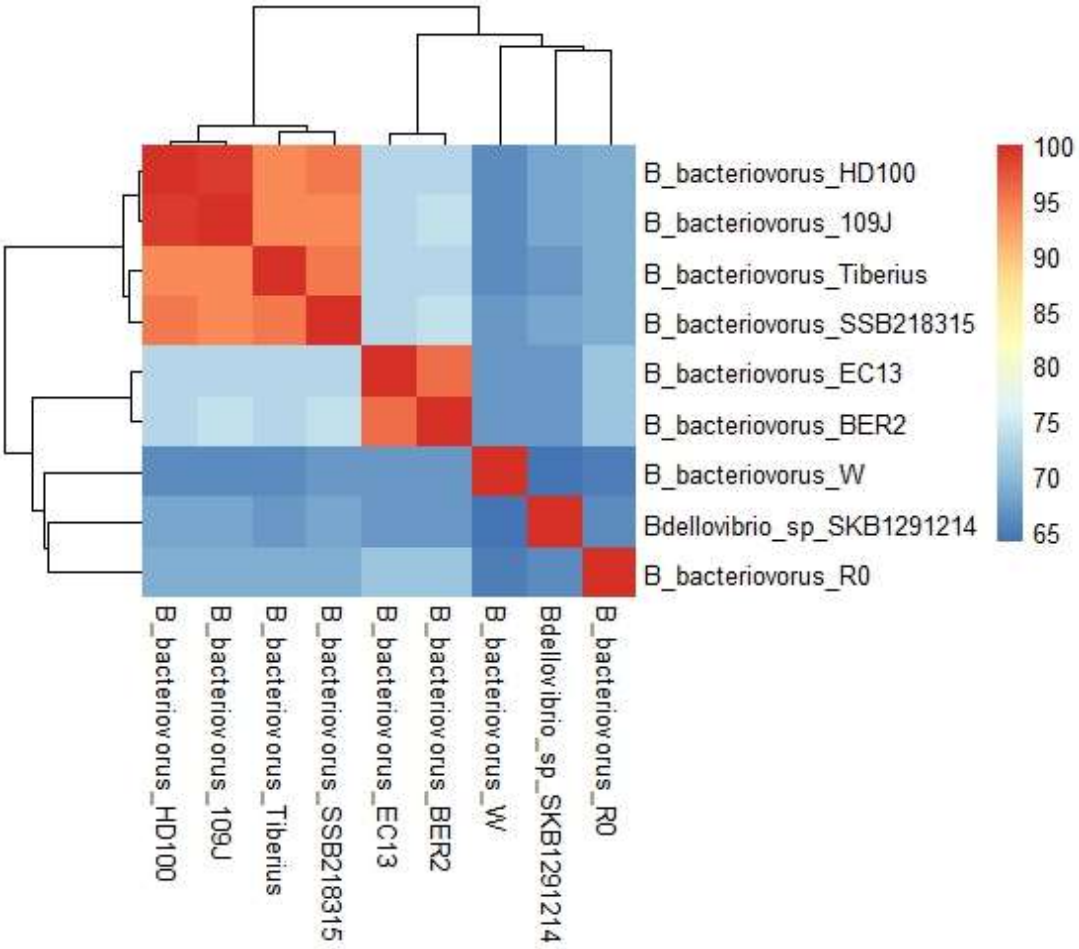


Figure 5. 10. Heatmap derived from the average amino acid identity matrix clustering analysis of the whole genomes of nine *Bdellovibrio* strains. Colours represent bands of percent identity. The heatmap was generated in R package plots using heatmap.2 function.

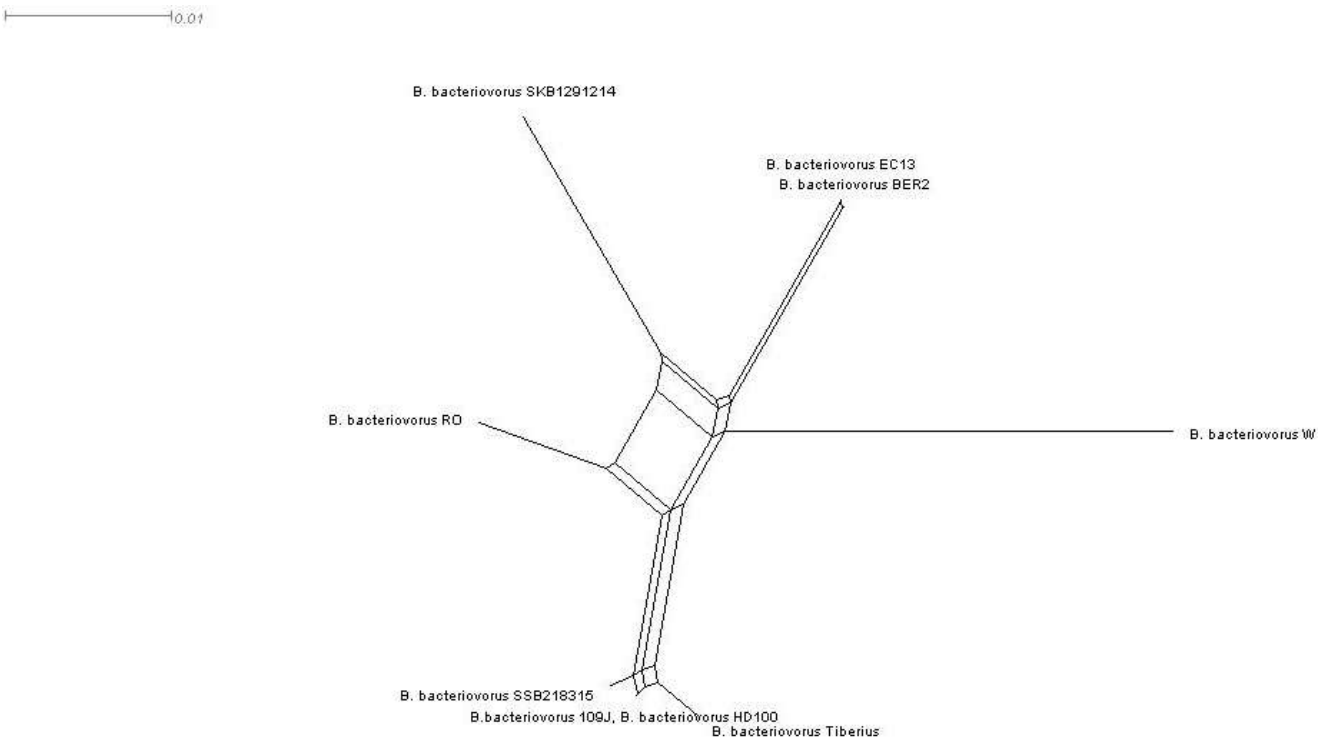


Figure 5. 11. Phylogenetic network showing the clustering pattern of the nine Bdellovibrio strains based on the 16S rRNA gene retrieved from each genomes. The network was constructed using SplitsTree 4.

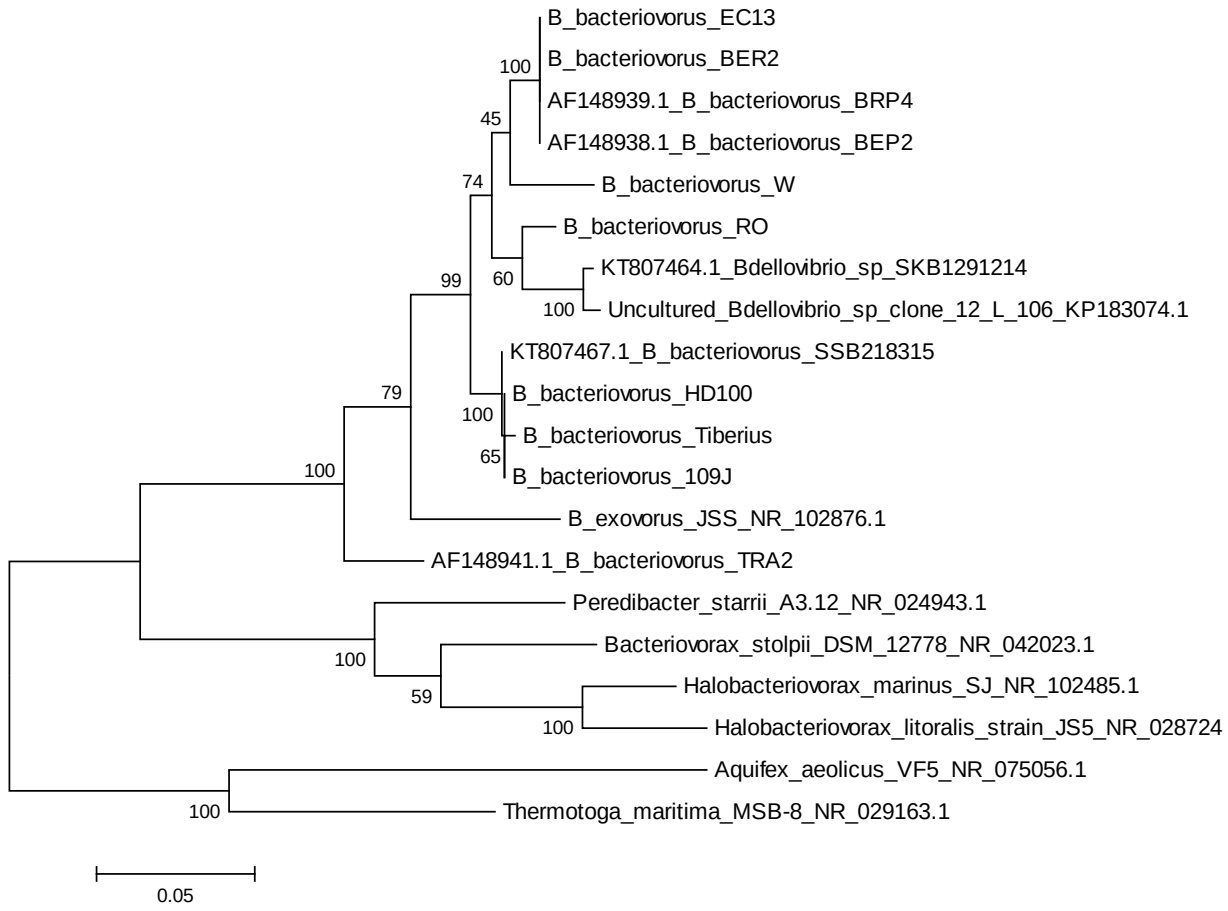


Figure 5. 12. Molecular Phylogenetic analysis by Maximum Likelihood method using 16S rRNA gene sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model with 1000 bootstrap replications. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 20 16S rRNA gene nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

AF148941.1_B_bacteriovorus_TRA2																			
AF148938.1_B_bacteriovorus_BEP2	0.071																		
AF148939.1_B_bacteriovorus_BRP4	0.071	0.000																	
KT807467.1_B_bacteriovorus_SSB218315	0.068	0.030	0.030																
KT807464.1_Bdellovibrio_sp_SKB1291214	0.085	0.034	0.034	0.043															
B_bacteriovorus_W	0.090	0.036	0.036	0.045	0.051														
B_bacteriovorus_Tiberius	0.073	0.034	0.034	0.004	0.047	0.048													
B_bacteriovorus_RO	0.077	0.033	0.033	0.028	0.033	0.044	0.031												
B_bacteriovorus_HD100	0.069	0.031	0.031	0.001	0.044	0.045	0.003	0.028											
B_bacteriovorus_109J	0.069	0.031	0.031	0.001	0.044	0.045	0.003	0.028	0.000										
B_bacteriovorus_EC13	0.071	0.000	0.000	0.030	0.034	0.036	0.034	0.033	0.031	0.031									
B_bacteriovorus_BER2	0.071	0.000	0.000	0.030	0.034	0.036	0.034	0.033	0.031	0.031	0.000								
B_exovorus_JSS_NR_102876.1	0.093	0.080	0.080	0.072	0.092	0.085	0.075	0.083	0.071	0.071	0.080	0.080							
Bacteriovorax_stolpii_DSM_12778_NR_042023.1	0.194	0.221	0.221	0.213	0.217	0.214	0.213	0.224	0.212	0.212	0.221	0.221	0.207						
Peredibacter_starrii_A3.12_NR_024943.1	0.197	0.215	0.215	0.214	0.221	0.217	0.219	0.222	0.215	0.215	0.215	0.215	0.211	0.119					
Halobacteriovorax_marinus_SJ_NR_102485.1	0.202	0.219	0.219	0.211	0.214	0.217	0.216	0.225	0.212	0.212	0.219	0.219	0.212	0.117	0.136				
Aquifex_aeolicus_VF5_NR_075056.1	0.314	0.338	0.338	0.344	0.351	0.341	0.344	0.346	0.345	0.345	0.338	0.338	0.333	0.342	0.351	0.337			
Thermotoga_maritima_MSB-8_NR_029163.1	0.259	0.270	0.270	0.270	0.289	0.277	0.273	0.279	0.270	0.270	0.270	0.270	0.269	0.272	0.300	0.290	0.224		
Uncultured_Bdellovibrio_sp_clone_12_L_106_KP183074.1	0.087	0.036	0.036	0.046	0.008	0.054	0.051	0.034	0.047	0.047	0.036	0.036	0.098	0.224	0.225	0.219	0.353	0.294	
Halobacteriovorax_litoralis_strain_JS5_NR_028724	0.208	0.228	0.228	0.225	0.227	0.230	0.226	0.232	0.225	0.225	0.228	0.228	0.211	0.117	0.147	0.068	0.344	0.305	0.234

Table 5. 4. Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 20 16S rRNA gene nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

5.6. Analysis of Orthologous Gene Clusters

From the comparative genome analysis using an Orthovenn diagram, a total of 3823 gene clusters were formed out of which 3778 were orthologous gene clusters shared by at least two species (Figure 5. 13). The six *Bdellovibrio* strains shared 1936 orthologous clusters as core genomes indicating their conservation among the six *Bdellovibrio* strains after speciation. The 1936 core genomes were made up of 1910 single-copy and 23 duplicated gene clusters i.e. the paralogs. A total of 45 clusters were found in only one strain, with strain SKB1291214 possessing thirty which is the highest number of unique clusters in a strain (Figure 5. 14). The ontology of these unique clusters includes proteins involved in molecule (ion, nucleic acid, nucleoside, nucleotide, and cofactor) binding, transporter, transferase, signal transducers, oxidoreductase, and hydrolase activities (Figure 5. 15). The unique clusters also contain sequences that have homology with protein (AmsF) involved in Amylovoran biosynthesis. BLAST analysis of the cluster sequences against the genome of *Bdellovibrio* sp. SKB1291214 in RAST showed the amylovoran biosynthesis protein AmsF region is associated with Autographivirinae Erwinia phage (Figure 5. 16).

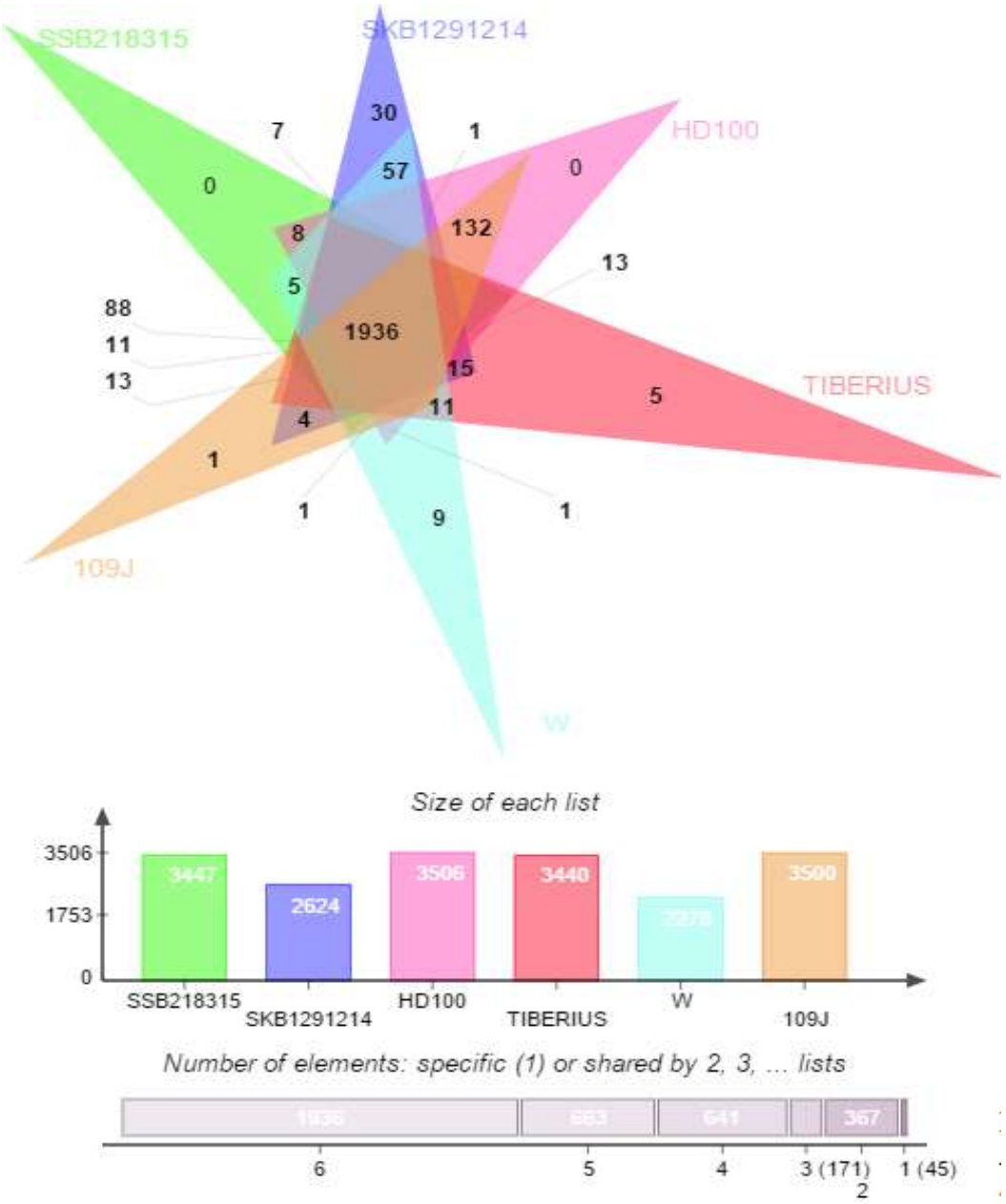


Figure 5. 13. Venn diagram showing unique and shared orthologous gene families between *Bdellovibrio* species. *Bdellovibrio* sp. SKB1291214, *B. bacteriovorus* SSB218315, *B. bacteriovorus* HD100 (BX842601), *B. bacteriovorus* Tiberius (CP002930), *B. bacteriovorus* 109J (CP007656), *B. bacteriovorus* W (CP002190). Orthologous gene families were identified using the web server OrthoVenn. (http://probes.pw.usda.gov/OrthoVenn)

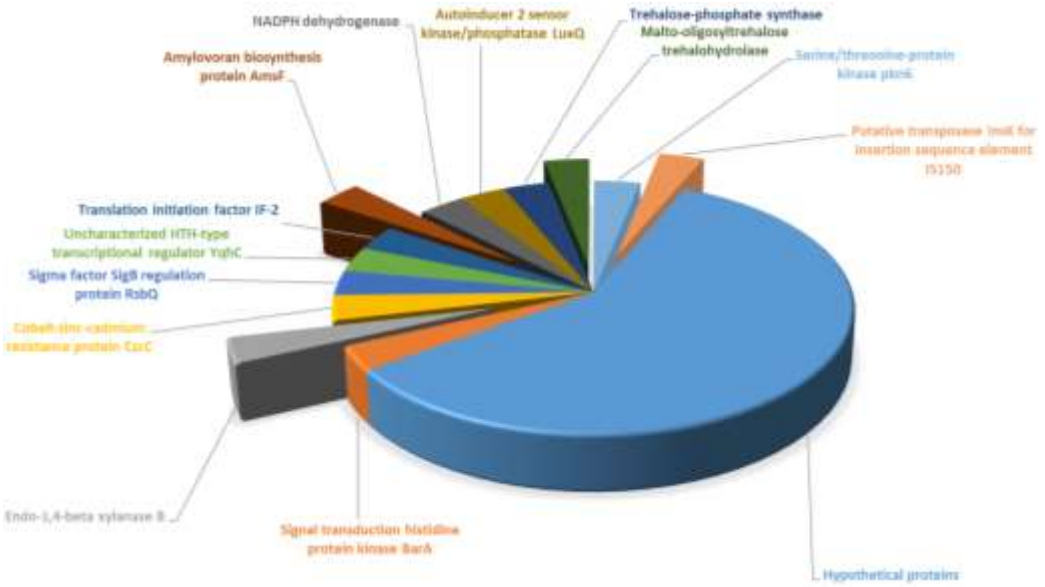


Figure 5. 14. The thirty unique gene clusters of *Bdellovibrio* sp. SKB1291214

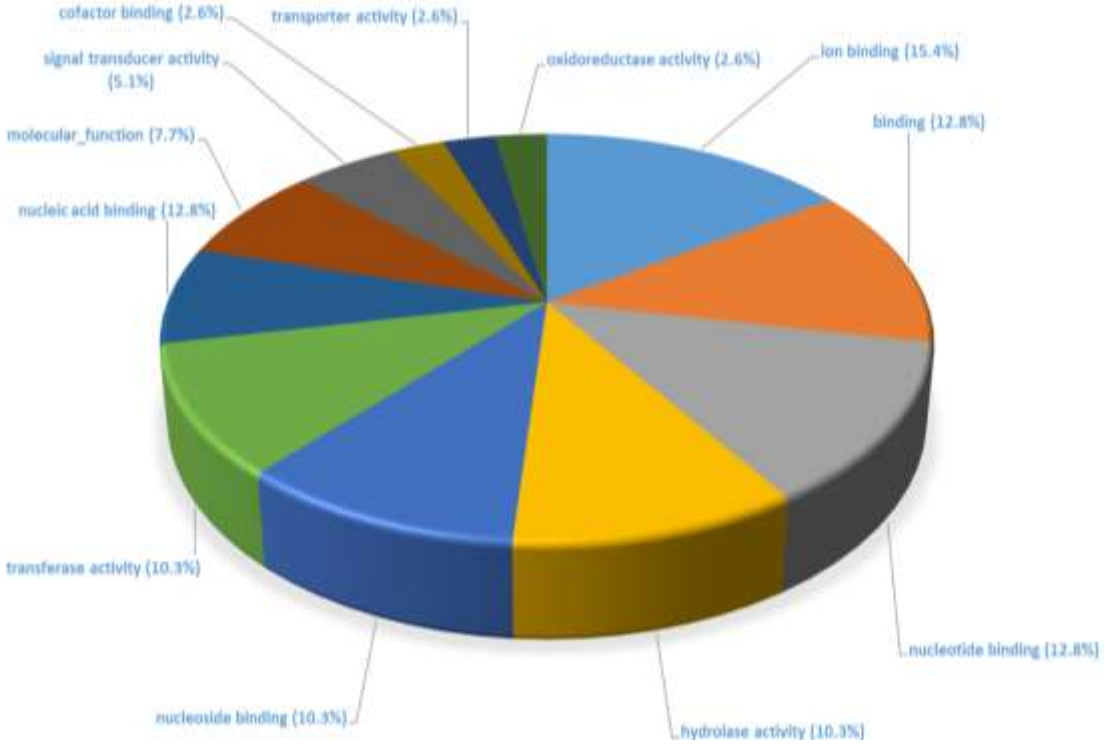


Figure 5. 15. **The ontology of the thirty unique gene clusters of *Bdellovibrio* sp. SKB1291214**

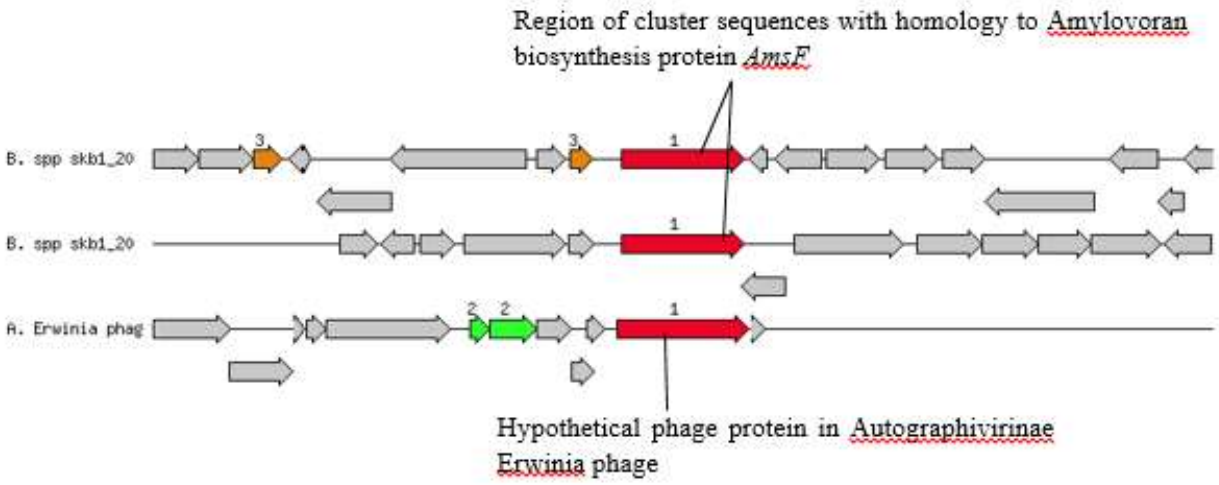


Figure 5. 16. The BLAST analysis of the cluster sequence Amylovoran biosynthesis protein AmsF against the genome of Bdellovibrio sp. SKB1291214 using the RAST online server.

5.7. Analysis of the host interaction (*hit*) locus.

Genomic maps were constructed to compare the *hit* locus and the adjoining genes between *Bdellovibrio* sp SKB1291214 and *B. bacteriovorus* SSB218315 (Figure 5. 17). The *hit* locus region was observed to be present in *B. bacteriovorus* SSB218315 based on successful PCR amplification of the region and significant e-value result obtained from BLASTp search in the NCBI database and ExPASy Bioinformatics Resource Portal. On the contrary, the BLASTp analysis of the region corresponding to the *hit* locus in SKB1291214 revealed sequence similarity with class I SAM-dependent methyltransferase found in *Bradyrhizobium* sp IS2118, however with an insignificant identity and e-value of 38 % and 5.9 respectively at the e-value threshold of 1e-6. The BLASTp analysis in the ExPASy Bioinformatics Resource Portal, however, gave no hit result.

Multiple sequence alignment of this region with other *Bdellovibrio* strains further revealed high sequence variation in the region corresponding to *Bd0108* in *Bdellovibrio* sp. SKB1291214 (Figure 5. 18). Furthermore, an additional fragment of 174 bp observed to be absent in *B. bacteriovorus* SSB218315 was found in *Bdellovibrio* sp. SKB1291214. This fragment was inserted between gene *cheY/Bd0103* and region

tagged *Bdt_0100* in the genome of *B. bacteriovorus* strain Tiberius, and produced an insignificant e-value with BLASTp search. The *Bd0109* gene part of the *hit* locus was observed to be conserved in all the *Bdellovibrio* spp. including *Bdellovibrio* sp. SKB1291214 and even *B. exovorus* (Figure 5. 19).

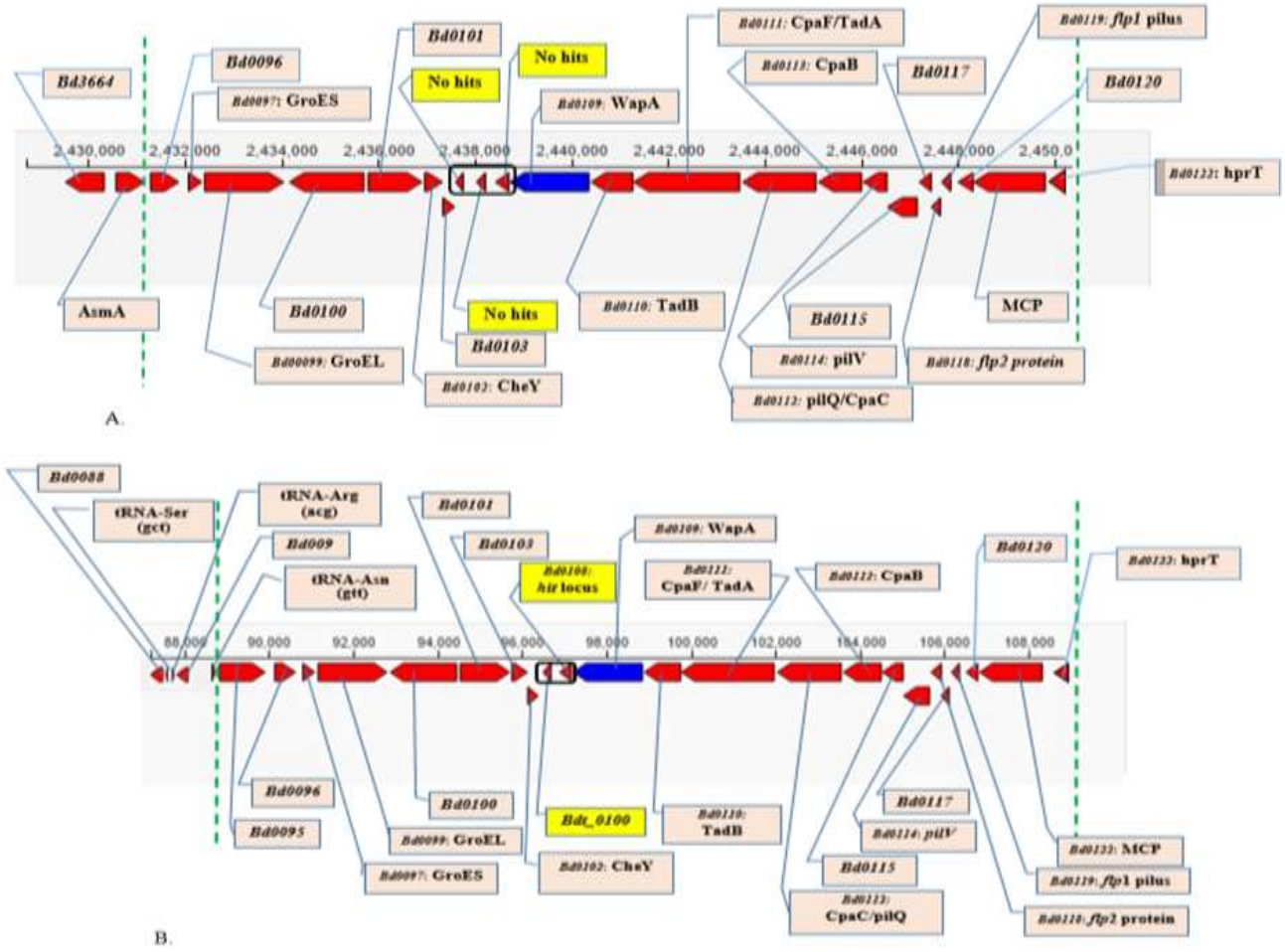


Figure 5. 17. Diagrammatic comparison of the *hit* locus and the adjoining regions between SKB1291214 (A.) and SSB218315 (B.). The major differences can be observed at the region after the *wapA* gene (circularized in black). There was no BLAST hits for *hit* locus orf4 (Bd0108), Bdt_0100 and another unidentified gene in *Bdellovibrio* sp. SKB1291214. Images was generated using KBase software (http://biorxiv.org/content/early/2016/12/22/096354) and BLAST analysis was done in ExPASy Bioinformatics Resource Portal (http://www.expasy.org/). hprT: Hypoxanthine-guanine phosphoribosyltransferase; pilus assembly protein (CpaB, CpaF/TadA, TadB, pilQ/Cpac, pilV, flp1, flp2); Chemotaxis protein (MCP: Methylaccepting chemotaxis protein, chemotaxis protein CheY), Heat-shock protein (GroES and GroEL), cell wall associated protein (wapA), host interaction (*hit*) locus orf4.

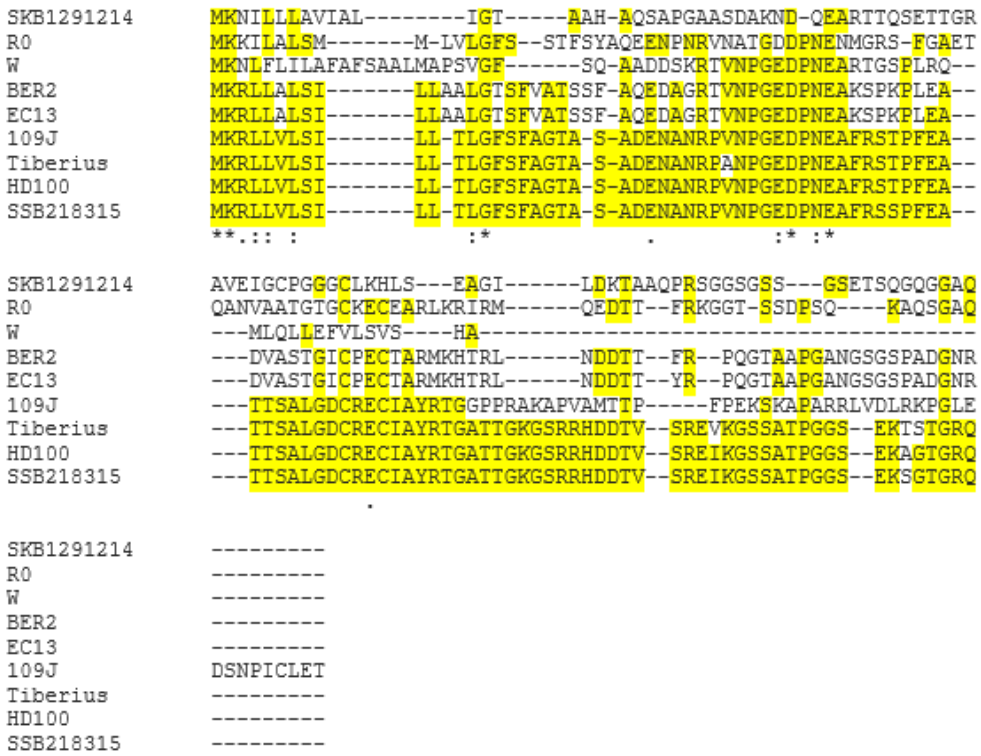


Figure 5. 18. Multiple sequence alignment of the *Bd0108* gene of *hit* locus using CLUSTAL O (1.2.4)

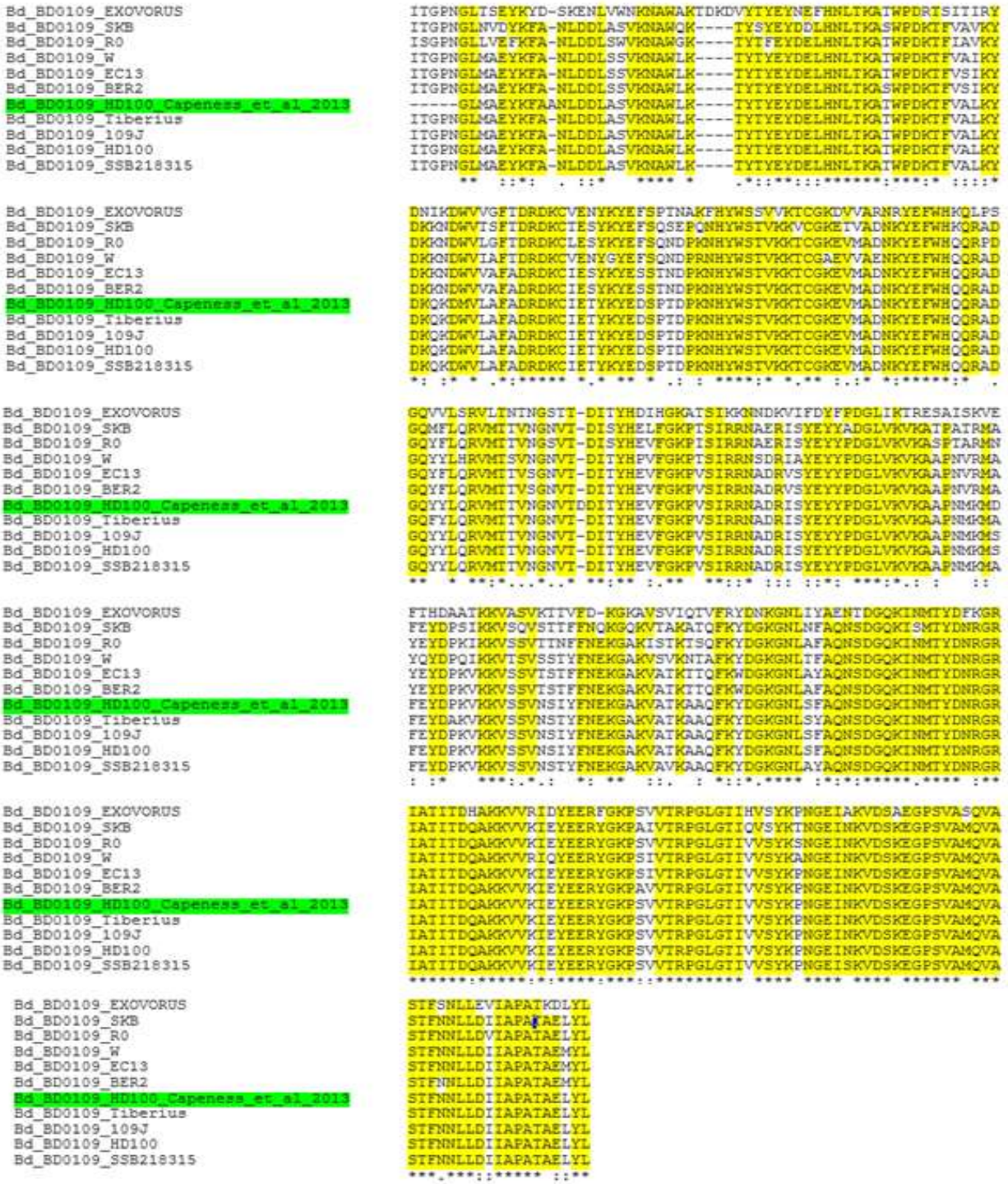


Figure 5. 19. Multiple sequence alignment of *Bd0109* gene of the *hit* locus using CLUSTAL O (1.2.4). The amino acid sequence of *Bd0109* reported by Capeness *et al*., 2013 was aligned with *Bd0109* region obtained from the other *Bdellovibrio* strains.

5.8. Genome Alignment and Visualization of *Bdellovibrio* spp.

The whole genome alignment and visualization of nine different *Bdellovibrio* spp. is presented in Figure 5. 20, 5. 21 and 5. 22. The MAUVE and the BRIGS analysis provided a diagrammatic view of the homology that exists in the genome sequence of the *Bdellovibrio* spp. considered for the analysis. Conserved synteny with similar gene orientation was observed in *Bdellovibrio* spp SSB218315, HD100 and Tiberius (Figure 5. 21). The genome of *Bdellovibrio* sp. SKB1291214 is characterized with rearrangements and inverisons of the LCB. The BLAST Ring Image Generator was used to generate a circular map that revealed the similarities that exist among the nine *Bdellovibrio* genomes in reference to *B. bacteriovorus* SSB218315 (Figure 5. 22). The gaps represent regions of the genome that are not identical in the *Bdellovibrio* strains. These gaps could be as a result of the presence of genomic islands.

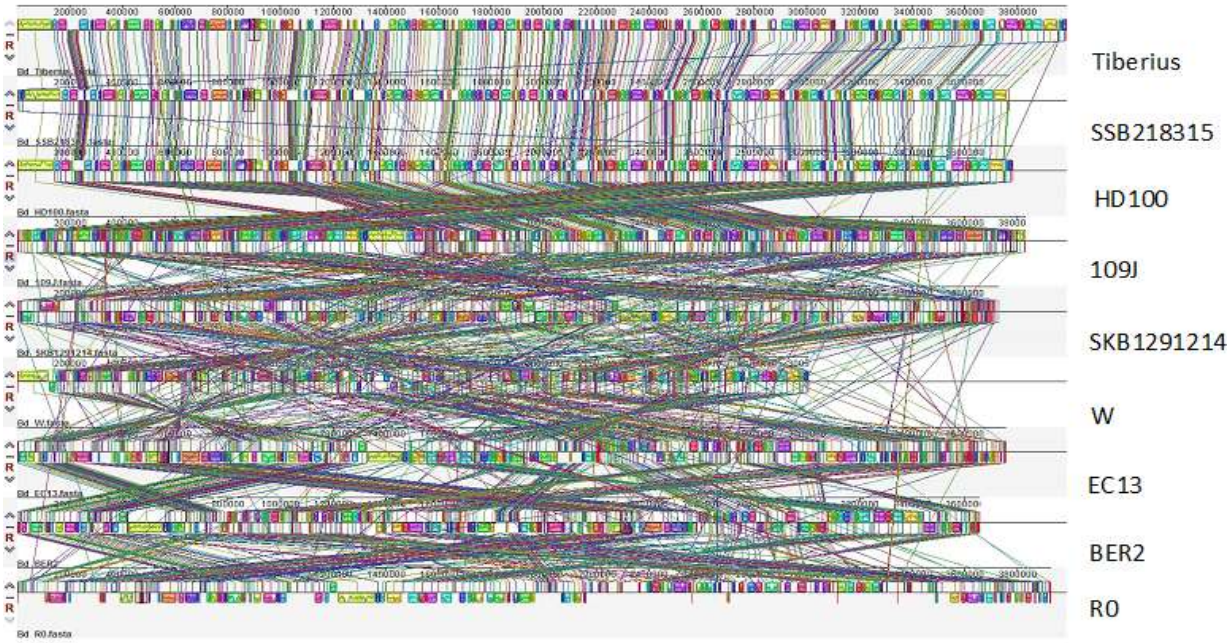


Figure 5. 20. Whole genome alignment of nine *Bdellovibrio* spp. constructed using MAUVE software.

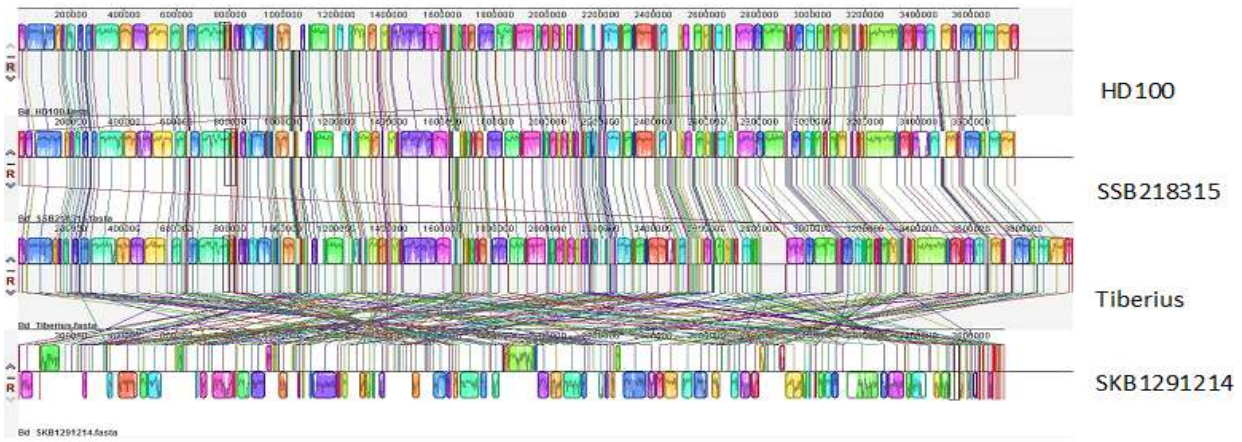


Figure 5. 21. Whole genome alignment of nine *Bdellovibrio* spp. constructed using MAUVE software. The conserved synteny among *B. bacteriovorus* strains HD100, SSB218315 and Tiberius can be observed.

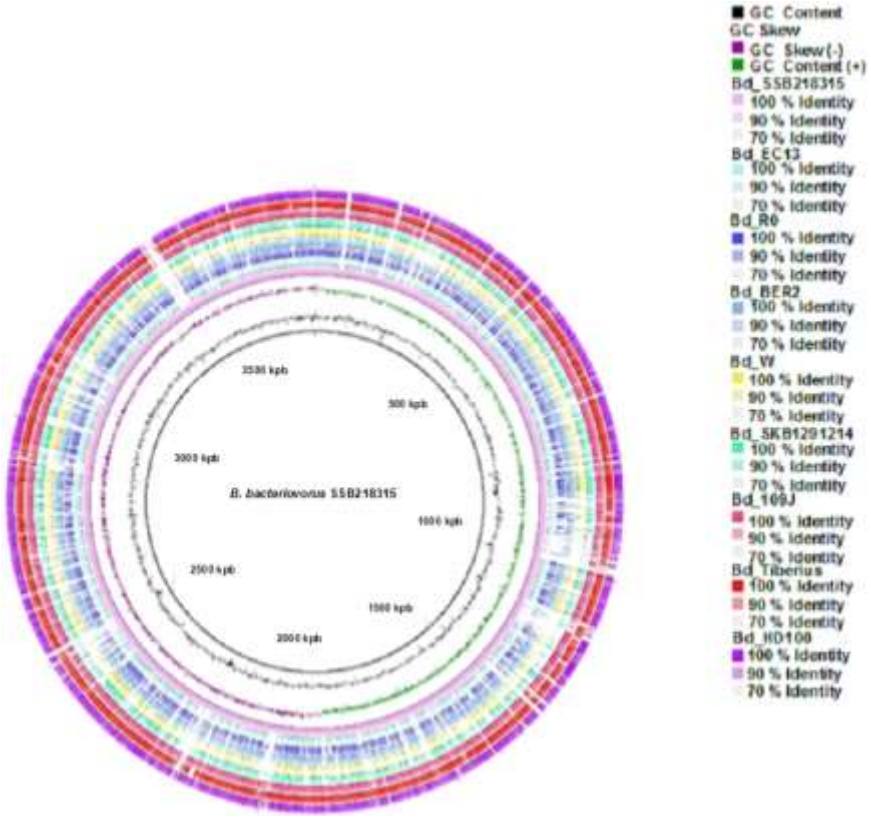


Figure 5. 22. Whole genome alignment of nine *Bdellovibrio* spp. constructed using BLAST Ring Image Generator (BRIG) with *B. bacteriovorus* SSB218315 serving as the reference.

5.9. Nucleotide Sequence Accession Numbers.

The whole genome shotgun project has been deposited at DDBJ/ENA/GenBank databases under the accession NELQ00000000 for *Bdellovibrio* sp. SKB1291214 (the version described in this paper is version NELQ01000000). The complete genome sequence of *B. bacteriovorus* SSB218315 was deposited in the same databases under accession number CP020946.

DISCUSSION

The genome sizes of the *Bdellovibrio* spp strains SKB1291214 and SSB218315 reported in this study are within the ranges observed for *Bdellovibrio* strains reported in earlier studies (Hobley *et al*., 2012; Rendulic *et al*., 2004). Being obligate predatory bacteria, *Bdellovibrio* spp. are expected to possess reduced genome sizes just as observed in other obligate intracellular parasites and prokaryotes such as phages and chlamydia (Sakharkar *et al*., 2004; Toft and Andersson, 2010). However in comparison to other predatory intracellular parasites, the *Bdellovibrio* spp. have relatively large genome sizes. This may be because their genomes code for "prey-seeking" factors and wide varieties of enzyme needed to find and attack their prey respectively. The ability of *Bdellovibrio* spp to switch from predatory lifestyle to growing axenically or saprophytically on nutrient-rich media could also be responsible for their large genome sizes (Seidler and Starr, 1969; Dashiff and Kadouri, 2009; Hobley *et al*., 2012).

Genomic islands acquired via Horizontal Gene Transfer (HGT) are known to confer special adaptive characteristics such as antibiotic resistance, survival features and metabolic activities such as metabolism of complex compounds (Juhas *et al*., 2009). Some distinguishing features of GEIs include association with genes coding for tRNA, integrase or transposase, and possession of percentage G+C content that is different from another part of the genome (Vernikos and Parkhill, 2008). Predicted GEIs of *Bdellovibrio* strains SKB1291214 and SSB218315 included a number of hypothetical proteins, peptidase, septation protein spoVG (in *B. bacteriovorus* SSB218315) and survival protein *surA* that ensures the survival of bacteria at the stationary growth phase. In addition, three genes encoding integrase usually associated with horizontal gene transfer were found among the predicted genomic islands in *Bdellovibrio* sp. SKB1291214.

The ontology of the thirty unique clusters revealed that they are rich in proteins involved in ion binding and hydrolase (Endo, 1, 4, beta-xylanase; COG0657: Acetyl esterase/lipase superfamily and trehalose hydrolase) activities that could enhance the predatory capabilities and nutrient acquisition of *Bdellovibrio* sp. SKB1291214. Furthermore, the unique gene clusters also contain transposases and Autographivirinae Erwinia phage associated region coding for protein *AmsF* involved in Amylovoran biosynthesis. The *AmsF* protein has been described to carry a secretory signal sequence involved in the periplasmic processing of

Amylovoran (Bugert and Geider, 1995), an exopolysaccharide that plays an important role in the virulence of *Erwinia Carotovora* (Koczan *et al*., 2009). *Bdellovibrio* sp. SKB1291214 has experienced horizontal gene transfer (HGT) which is evident with the presence of integrases among the GEIs, transposases among its unique gene clusters and the phage associated protein *AmsF*. BLASTp analysis of some of the unique gene cluster using an e-value threshold of 1e-6 revealed that SKB1291214 acquired some of these genes from bacteria that belong to groups other than class deltaproteobacteria (alphaproteobacteria, betaproteobacteria, and gammaproteobacteria). It is, however, pertinent to study and understand the extent and frequency of HGT in *Bdellovibrio* spp. to ensure their successful application as biocontrol agents.

The factors that can aid predation in *Bdellovibrio* spp. found in the genomes of the *Bdellovibrio* strains include flagellar, Type IV pilus, chemotaxis and toxin associated factors. They also possess genes coding for several serine proteases, DNases, RNases, hydrolases, lipases, peptidase, esterases, and siderophores. All these factors are important in prey location, attachment, invasion, digestion, and release of *Bdellovibrio* spp. progenies from exhausted prey cells. *Bdellovibrio* spp. have been described to be non-pathogenic to human (Gupta *et al*., 2016). However, genomes of *Bdellovibrio* spp. SKB129124, SSB218315, and other *Bdellovibrio* strains encode genes for collagenase and hemolysin, virulence factors that have been associated with some pathogens of human such as *Staphylococcus auereus* (Otto, 2014) and *Vibrio vulnificus* (Miyoshi, 2013). In addition, the genome of *B. bacteriovorus* SSB218315 also encodes gene for RTX toxins. This factor has been described to have different biological functions such as pore forming leukotoxin, metalloprotease and lipase activities (Linhartová *et al*., 2010). It is very important to characterize and identify the biological roles of these virulence factors in *Bdellovibrio* spp. so that they will not be a hindrance to their successful application as biocontrol agents of pathogens in humans.

For strains to belong to the same species, they must have ANI and AAI ≥ 95 %, <10 Karlin genomic signature and > 70 % *in silico* GGDH (Konstantinidis and Tiedje, 2007). The AAI between strain SKB1291214 and other strains was very low (63.70 – 67.68 %) while strain SSB218315 shared high AAI value of 95 % with *B. bacteriovorus* strains HD100, Tiberius and 109J. The whole genome alignment using MAUVE and BRIGS further showed the conserved synteny that exists among these strains. The implication

of this is that strain SSB218315 is closely related with HD100, Tiberius, and 109J and can conveniently be grouped together as same species. Meanwhile considering the percentage GC content, phylogenetic tree clustering pattern and AAI value, strain SKB1291214 could be grouped as a novel species.

The *hit* locus has been described to be made up of gene tagged *Bd0108* and part of gene tagged *Bd0109* in *B. bacteriovorus* HD100. The *Bd0109* gene part of the *hit* locus was observed to be conserved in all the *Bdellovibrio* spp. including *Bdellovibrio* sp. SKB1291214 and even *B. exovorus*. The implication of this observation is that *Bd0109* gene is very important in the predatory activities of *Bdellovibrio* spp. while variations in the sequence of *Bd0108* is not sufficient to hinder predation in *Bdellovibrio* spp. However, the variation in the sequence of *Bd0108* might have an influence on the predatory activities of *Bdellovibrio* spp. such as elongation of the period of attack (plaque formation) as well as a reduction in prey range as observed in *Bdellovibrio* sp. SKB1291214 (Oyedara *et al*., 2016). Mutation at the *hit* locus has been described to be responsible for the conversion of *Bdellovibrio* spp. to the host-independent phenotype (Capeness *et al*., 2013). Several attempts to grow *Bdellovibrio* sp. SKB1291214 in the absence of prey on nutrient-rich media for possible comparison with the predatory phenotype has not been successful.

PERSPECTIVES

The potential applications of *Bdellovibrio* spp. are enormous and for the first time in Mexico, we presented report on isolation *Bdellovibrio* spp. in Mexico. In this study, we further discussed the different fields in

Mexico where these versatile bacteria can be applied viz medicine, agriculture, food industry, animal husbandry, sewage treatment, and industries involved in biorecovery of compounds. The isolation, propagation and Laboratory maintenance procedures for *Bdellovibrio* spp is well documented in this study. More research studies on the applications of *Bdellovibrio* spp. can, therefore, stem from this scientific report. Studies can also be directed towards how mass production of *Bdellovibrio* spp. can be accomplished for application purposes.

Bdellovibrio spp. are not prey-specific, and therefore can use any susceptible prey as "food" including beneficial bacteria. Studies that is focused on understanding the predator-prey interaction of *Bdellovibrio* spp. will be essential to raise strains of *Bdellovibrio* sp. can be specifically used to attack a specific pathogen of interest. Furthermore, it will also be interesting to study the rate and pattern of *Bdellovibrio* predacity when more than one strain of *Bdellovibrio* spp are used in a "cocktail manner" against mixtures of pathogens. Efforts can also be intensified towards successful application of *Bdellovibrio* spp against Gram-positive bacteria since there is evidence that they can also prey on Gram-positive bacteria via an epibiotic mode of attack.

We also showed in this study that genome of *Bdellovibrio* spp. houses plethora of enzymes for predation. Some of these enzymes can be extracted and used for industrial purposes. Moreover, successful isolation of this prey-independent strains will also help in further understanding the predatory nature of *Bdellovibrio* spp. This report also showed evidence of horizontal gene transfer in *Bdellovibrio* spp. Wet Lab studies can be carried out to reveal the frequency at which *Bdellovibrio spp* acquire foreign DNA, and provide insight into how this horizontal gene transfer can shape the genome of *Bdellovibrio* spp. or hinder its applications.

Finally, we were able to isolate *Bdellovibrio* strains that exhibited genomic variations from soil samples in Reynosa, Tamaulipas, Mexico thereby confirming the existence of heterogeneity among strains of *Bdellovibrio* spp. Taxonomic review of the genus *Bdellovibrio* will be essential to further delineate the members into different taxonomic group.

REFERENCES

Al-Hissnawy, D.S., Al-Jibouri, S.A., AL-Thahab, A.A., Hameed, A.M., AL-Hijia, N.N., Al-Azzawi, A.A., Al-Umeri, K.K., Al-Khafaji, T.J., Raheem, R.M. and Al-Khafaji, J.K.T., 2012. Evaluation of *Citrobacter freundii* as a Heat Labile (LT) Enterotoxin Producer. *ltA*, *94*(5), p.94.

Alikhan, N.F., Petty, N.K., Zakour, N.L.B. and Beatson, S.A., 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC genomics*, *12*(1), p.402.

Aminov, R.I., 2011. Horizontal gene exchange in environmental microbiota. *Frontiers in microbiology*, *2*.

Argaw, A. and Mnalku, A., 2017. Symbiotic effectiveness of *Rhizobium leguminosarum* bv. vicieae isolated from major highland pulses on field pea (Pisum sativum L.) in soil with abundant rhizobial population. *Annals of Agrarian Science*.

Atterbury, R.J., Hobley, L., Till, R., Lambert, C., Capeness, M.J., Lerner, T.R., Fenton, A.K., Barrow, P. and Sockett, R.E., 2011. Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. *Applied and environmental microbiology*, *77*(16), pp.5794-5803.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M. and Meyer, F., 2008. The RAST Server: rapid annotations using subsystems technology. *BMC genomics*, *9*(1), p.75.

Baer, M.L., Ravel, J., Chun, J., Hill, R.T. and Williams, H.N., 2000. A proposal for the reclassification of *Bdellovibrio stolpii* and *Bdellovibrio starrii* into a new genus, *Bacteriovorax* gen. nov. as *Bacteriovorax stolpii* comb. nov. and *Bacteriovorax starrii* comb. nov., respectively. *International journal of systematic and evolutionary microbiology*, *50*(1), pp.219-224.

Baer, M.L., Ravel, J., Piñeiro, S.A., Guether-Borg, D. and Williams, H.N., 2004. Reclassification of salt-water *Bdellovibrio* sp. as *Bacteriovorax marinus* sp. nov. and *Bacteriovorax litoralis* sp. nov. *International journal of systematic and evolutionary microbiology*, *54*(4), pp.1011-1016.

Bai, L., Xia, S., Lan, R., Liu, L., Ye, C., Wang, Y., Jin, D., Cui, Z., Jing, H., Xiong, Y. and Bai, X., 2012. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. *PLoS One*, *7*(3), p.e33054.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D. and Pyshkin, A.V., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology*, *19*(5), pp.455-477.

Barel, G. and Jurkevitch, E., 2001. Analysis of phenotypic diversity among host-independent mutants of Bdellovibrio bacteriovorus 109J. Archives of microbiology, 176(3), pp.211-216.

Boileau, M.J., Clinkenbeard, K.D. and Iandolo, J.J., 2011. Assessment of *Bdellovibrio bacteriovorus* 109J killing of *Moraxella bovis* in an in vitro model of infectious bovine keratoconjunctivitis. *Canadian Journal of Veterinary Research*, *75*(4), pp.285-291.

Boileau, M.J., Mani, R., Breshears, M.A., Gilmour, M., Taylor, J.D. and Clinkenbeard, K.D., 2016. Efficacy of *Bdellovibrio bacteriovorus* 109J for the treatment of dairy calves with experimentally induced infectious bovine keratoconjunctivitis. *American journal of veterinary research*, *77*(9), pp.1017-1028.

Bosi, E., Donati, B., Galardini, M., Brunetti, S., Sagot, M.F., Lió, P., Crescenzi, P., Fani, R. and Fondi, M., 2015. MeDuSa: a multi-draft based scaffolder. *Bioinformatics*, *31*(15), pp.2443-2451.

Bugert, P. and Geider, K., 1995. Molecular analysis of the ams operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Molecular microbiology*, *15*(5), pp.917-933.

Burnham, J. C., and Robinson, J., 1974. Genus *Bdellovibrio* Stolp and Starr. In Bergey's Manual of determinative bacteriology. 8th ed. Edited by R. E. Buchanan and N. E. Gibbons. *The Williams and Wilkins Co. Baltimore*, pp. 212-214.

Burnham, J.C., Stetak, T. and Locher, G., 1976. Extracellular lysis of the bluegreen alga *Phormidium luridum* by *Bdellovibrio bacteriovorus*. *Journal of Phycology*, *12*(3), pp.306-313.

Cao, H., An, J., Zheng, W. and He, S., 2015. *Vibrio cholerae* pathogen from the freshwater-cultured whiteleg shrimp *Penaeus vannamei* and control with *Bdellovibrio bacteriovorus*. *Journal of invertebrate pathology*, *130*, pp.13-20.

Cao, H., He, S., Lu, L., Yang, X. and Chen, B., 2014. Identification of a *Proteus penneri* isolate as the causal agent of red body disease of the cultured white shrimp *Penaeus vannamei* and its control with *Bdellovibrio bacteriovorus. Antonie van Leeuwenhoek*, *105*(2), pp.423-430.

Cao, H., He, S., Wang, H., Hou, S., Lu, L. and Yang, X., 2012. Bdellovibrios, potential biocontrol bacteria against pathogenic *Aeromonas hydrophila*. *Veterinary microbiology*, *154*(3), pp.413-418.

Capeness, M.J., Lambert, C., Lovering, A.L., Till, R., Uchida, K., Chaudhuri, R., Alderwick, L.J., Lee, D.J., Swarbreck, D., Liddell, S. and Aizawa, S.I., 2013. Activity of Bdellovibrio hit locus proteins, Bd0108 and Bd0109, links Type IVa pilus extrusion/retraction status to prey-independent growth signalling. Plos one, 8(11), p.e79759.

Carver, T., Thomson, N., Bleasby, A., Berriman, M. and Parkhill, J., 2008. DNAPlotter: circular and linear interactive genome visualization. *Bioinformatics*, *25*(1), pp.119-120.

Chaudari, R.P., Ahire, D.V., Chkravarty, M. and Maity, S., 2014. Electrical conductivity as a tool for determining the physical properties of indian soils. *International Journal of Scientific and Research Publications*, *4*(4), pp.1-4.

Chen, L., Xiong, Z., Sun, L., Yang, J. and Jin, Q., 2011. VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic acids research*, *40*(D1), pp.D641-D645.

Chu, W.H. and Zhu, W., 2010. Isolation of *Bdellovibrio* as biological therapeutic agents used for the treatment of *Aeromonas hydrophila* infection in fish. *Zoonoses and public health*, *57*(4), pp.258-264.

Clinical Laboratory Standard Institute (2012). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. M100-S22. 32 (3).

Cotter, T.W. and Thomashow, M.F., 1992. A conjugation procedure for *Bdellovibrio bacteriovorus* and its use to identify DNA sequences that enhance the plaque-forming ability of a spontaneous host-independent mutant. Journal of bacteriology, 174(19), pp.6011-6017.

Cotter, T.W. and Thomashow, M.F., 1992. Identification of a *Bdellovibrio bacteriovorus* genetic locus, *hit*, associated with the host-independent phenotype. *Journal of bacteriology*, *174*(19), pp.6018-6024.

Darling, A.C., Mau, B., Blattner, F.R. and Perna, N.T., 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome research*, *14*(7), pp.1394-1403.

Dashiff, A. and Kadouri, D.E., 2009. A new method for isolating host-independent variants of *Bdellovibrio bacteriovorus* using E. coli auxotrophs. The open microbiology journal, 3, p.87.

Dashiff, A. and Kadouri, D.E., 2011. Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. *Molecular oral microbiology*, *26*(1), pp.19-34.

Dashiff, A., Junka, R.A., Libera, M. and Kadouri, D.E., 2011. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. *Journal of applied microbiology*, *110*(2), pp.431-444.

Dashiff, A., Keeling, T.G. and Kadouri, D.E., 2011. Inhibition of predation by *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* via host cell metabolic activity in the presence of carbohydrates. *Applied and environmental microbiology*, *77*(7), pp.2224-2231.

Davidov, Y. and Jurkevitch, E., 2004. Diversity and evolution of *Bdellovibrio*-and-like organisms (BALOs), reclassification of *Bacteriovorax starrii* as *Peredibacter starrii* gen. nov., comb. nov., and description of the *Bacteriovorax–Peredibacter* clade as Bacteriovoracaceae fam. nov. *International journal of systematic and evolutionary microbiology*, *54*(5), pp.1439-1452.

Davidov, Y. and Jurkevitch, E., 2009. Predation between prokaryotes and the origin of eukaryotes. *BioEssays*, *31*(7), pp.748-757.

de Dios Caballero, J., Vida, R., Cobo, M., Máiz, L., Suárez, L., Galeano, J., Baquero, F., Cantón, R., del Campo, R. and GEIFQ Study Group, 2017. Individual Patterns of Complexity in Cystic Fibrosis Lung Microbiota, Including Predator Bacteria, over a 1-Year Period. *mBio*, *8*(5), pp.e00959-17.

Diedrich, D.L., Denny, C.F., Hashimoto, T. and Conti, S.F., 1970. Facultatively parasitic strain of Bdellovibrio bacteriovorus. Journal of bacteriology, 101(3), pp.989-996.

Dijkshoorn, L., Nemec, A. and Seifert, H., 2007. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. *Nature Reviews Microbiology*, *5*(12), pp.939-951.

Dwidar, M., Nam, D. and Mitchell, R.J., 2015. Indole negatively impacts predation by *Bdellovibrio bacteriovorus* and its release from the bdelloplast. *Environmental microbiology*, *17*(4), pp.1009-1022.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), pp.2460-2461.

Ekseth, O.K., Kuiper, M. and Mironov, V., 2013. orthAgogue: an agile tool for the rapid prediction of orthology relations. *Bioinformatics*, *30*(5), pp.734-736.

Eng, S.K., Pusparajah, P., Ab Mutalib, N.S., Ser, H.L., Chan, K.G. and Lee, L.H., 2015. *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*, *8*(3), pp.284-293.

Epton, H.A.S., Walker, N.M. and Sigee, D.C., 1989. *Bdellovibrio*: a potential control agent for soft rot and black leg of potato. *Plant pathogenic bacteria: part A. Akademia Kiado, Budapest, Hungary*, pp.207-212.

Evans, K.J., Lambert, C. and Sockett, R.E., 2007. Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *Journal of bacteriology*, *189*(13), pp.4850-4859.

Ferguson, M.A., Schmitt, J.L., Sindhurakar, A.R., Volle, C.B., Nuñez, M.E. and Spain, E.M., 2008. Rapid isolation of host-independent Bdellovibrio bacteriovorus. Journal of microbiological methods, 73(3), pp.279-281.

Fratamico, P.M. and Cooke, P.H., 1996. Isolation of Bdellovibrios that prey on *Escherichia coli* O157: H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. *Journal of food safety*, *16*(2), pp.161-173.

Fratamico, P.M. and Whiting, R.C., 1995. Ability of *Bdellovibrio bacteriovorus* 109J to lyse gram-negative food-borne pathogenic and spoilage bacteria. *Journal of food Protection*, *58*(2), pp.160-164.

Friedberg, D., 1978. Growth of host dependent Bdellovibrio in host cell free system. Archives of microbiology, 116(2), pp.185-190.

Fry, J.C. and Staples, D.G., 1976. Distribution of *Bdellovibrio bacteriovorus* in sewage works, river water, and sediments. *Applied and environmental microbiology*, *31*(4), pp.469-474.

Fulthorpe, R.R., Roesch, L.F., Riva, A. and Triplett, E.W., 2008. Distantly sampled soils carry few species in common. *The ISME journal*, *2*(9), p.901.

Gophna, U., Charlebois, R.L. and Doolittle, W.F., 2006. Ancient lateral gene transfer in the evolution of Bdellovibrio bacteriovorus. *Trends in microbiology*, *14*(2), pp.64-69.

Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P. and Tiedje, J.M., 2007. DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *International journal of systematic and evolutionary microbiology*, *57*(1), pp.81-91.

Guerrero, R., Pedrós-Alió, C., Esteve, I., Mas, J., Chase, D. and Margulis, L., 1986. Predatory prokaryotes: predation and primary consumption evolved in bacteria. *Proceedings of the National Academy of Sciences*, *83*(7), pp.2138-2142.

Guether, D.L. and Williams, H.N., 1993. Antibiogram characterization of aquatic and terrestrial *Bdellovibrio* isolates. Abstract Q-244 presented at the 93rd General Meeting of the American Society for Microbiology. *Atlanta, Georgia, USA*.

Guo, Y., Pan, Q., Yan, S., Chen, Y., Li, M., Chen, D., Han, H., Wu, B. and Cai, J., 2017. *Bdellovibrio* and like organisms promoted growth and survival of juvenile abalone *Haliotis discus hannai Ino* and modulated bacterial community structures in its gut. *Aquaculture International*, 25(4), pp.1625–1643.

Guo, Y., Yan, L. and Cai, J., 2016. Effects of *Bdellovibrio* and Like Organisms on Survival and Growth Performance of Juvenile Turbot, *Scophthalmus maximus*. *Journal of the World Aquaculture Society*, *47*(5), pp.633-645.

Gupta, S., Tang, C., Tran, M. and Kadouri, D.E., 2016. Effect of predatory bacteria on human cell lines. *PloS one*, *11*(8), p.e0161242.

Harini, K., Ajila, V. and Hegde, S., 2013. *Bdellovibrio bacteriovorus*: A future antimicrobial agent?. *Journal of Indian Society of Periodontology*, *17*(6), p.823.

Hayek, N., 2013. Lateral transfer and GC content of bacterial resistance genes. *Frontiers in microbiology*, *4*.

Hennequin, C. and Forestier, C., 2007. Influence of capsule and extended-spectrum beta-lactamases encoding plasmids upon *Klebsiella pneumoniae* adhesion. *Research in microbiology*, *158*(4), pp.339-347.

Hespell, R.B., Paster, B.J., Macke, T.J. and Woese, C.R., 1984. The origin and phylogeny of the bdellovibrios. *Systematic and applied microbiology*, *5*(2), pp.196-203.

Hespell, R.B., Rosson, R.A., Thomashow, M.F. and Rittenberg, S.C., 1973. Respiration of *Bdellovibrio bacteriovorus* strain 109J and its energy substrates for intraperiplasmic growth. *Journal of bacteriology*, *113*(3), pp.1280-1288.

Hobley, L., King, J.R. and Sockett, R.E., 2006. *Bdellovibrio* predation in the presence of decoys: three-way bacterial interactions revealed by mathematical and experimental analyses. *Applied and environmental microbiology*, *72*(10), pp.6757-6765.

Hobley, L., Lerner, T.R., Williams, L.E., Lambert, C., Till, R., Milner, D.S., Basford, S.M., Capeness, M.J., Fenton, A.K., Atterbury, R.J. and Harris, M.A., 2012. Genome analysis of a simultaneously predatory and prey-independent, novel Bdellovibrio bacteriovorus from the River Tiber, supports in silico predictions of both ancient and recent lateral gene transfer from diverse bacteria. *BMC genomics*, *13*(1), p.670.

Horcas, I., Fernández, R., Gomez-Rodriguez, J.M., Colchero, J., Gómez-Herrero, J.W.S.X.M. and Baro, A.M., 2007. WSXM: a software for scanning probe microscopy and a tool for nanotechnology. *Review of Scientific Instruments*, *78*(1), p.013705.

Horowitz, A.T., Kessel, M. and Shilo, M., 1974. Growth cycle of predacious Bdellovibrios in a host-free extract system and some properties of the host extract. Journal of bacteriology, 117(1), pp.270-282.

Hsiao, W., Wan, I., Jones, S.J. and Brinkman, F.S., 2003. IslandPath: aiding detection of genomic islands in prokaryotes. *Bioinformatics*, *19*(3), pp.418-420.

Huang, J.C.C. and Starr, M.P., 1973. Possible enzymatic bases of bacteriolysis by bdellovibrios. *Archiv für Mikrobiologie*, *89*(2), pp.147-167.

Ibragimov, F., 1980. Dissemination of *Bdellovibrio acteriovorus* in animals and their interaction with the agents of acute intestinal infections. *Zhurnal mikrobiologii, epidemiologii, i immunobiologii*, (5), pp.97-99.

Ikushiro, H., Islam, M.M., Tojo, H. and Hayashi, H., 2007. Molecular characterization of membrane-associated soluble serine palmitoyltransferases from *Sphingobacterium multivorum* and *Bdellovibrio stolpii*. *Journal of bacteriology*, *189*(15), pp.5749-5761.

Illumina Inc. (2009). Pair-End Sample preparation guide.

https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf

Illumina Inc. (2010). Illumina Sequencing Technology

http://prodata.swmed.edu/LepDB/Protocol/illumina_Paired-End_Sample_Preparation_Guide.pdf

Im, H., Son, S., Mitchell, R.J. and Ghim, C.M., 2017. Serum albumin and osmolality inhibit *Bdellovibrio bacteriovorus* predation in human serum. *Scientific Reports*, *7*:5896

Ishiguro, E.E., 1973. A growth initiation factor for host-independent derivatives of Bdellovibrio bacteriovorus. Journal of bacteriology, 115(1), pp.243-252.

Joshi, N.A. and Fass, J.N., 2011. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33)[Software].

Juhas, M., van der Meer, J.R., Gaillard, M., Harding, R.M., Hood, D.W. and Crook, D.W., 2009. Genomic islands: tools of bacterial horizontal gene transfer and evolution. *FEMS microbiology reviews*, *33*(2), pp.376-393.

Jurkevitch, E. and Davidov, Y., 2006. Phylogenetic diversity and evolution of predatory prokaryotes. In *Predatory Prokaryotes* (pp. 11-56). Springer Berlin Heidelberg.

Jurkevitch, E. and Ramati, B., 2000. Design and uses of *Bdellovibrio* 16S rRNA-targeted oligonucleotides. *FEMS microbiology letters*, *184*(2), pp.265-271.

Jurkevitch, E., 2012. Isolation and classification of *Bdellovibrio* and like organisms. *Current protocols in microbiology*, pp.7B-1.

Jurkevitch, E., Minz, D., Ramati, B. and Barel, G., 2000. Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio spp*. isolated on phytopathogenic bacteria. *Applied and environmental microbiology*, *66*(6), pp.2365-2371.

Kadouri, D.E., To, K., Shanks, R.M. and Doi, Y., 2013. Predatory bacteria: a potential ally against multidrug-resistant Gram-negative pathogens. *PloS one*, *8*(5), p.e63397.

Kelley, J.I. and Williams, H.N., 1992. Bdellovibrios in *Callinectus sapidus*, the blue crab. *Applied and environmental microbiology*, *58*(4), pp.1408-1410.

Kelley, J.I., Turng, B., Williams, H.N. and Baer, M.L., 1997. Effects of temperature, salinity, and substrate on the colonization of surfaces in situ by aquatic bdellovibrios. *Applied and environmental microbiology*, *63*(1), pp.84-90.

Kim, E.H., Dwidar, M., Mitchell, R.J. and Kwon, Y.N., 2013. Assessing the effects of bacterial predation on membrane biofouling. *Water research*, *47*(16), pp.6024-6032.

Koczan, J.M., McGrath, M.J., Zhao, Y. and Sundin, G.W., 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Phytopathology*, *99*(11), pp.1237-1244.

Konstantinidis, K.T. and Stackebrandt, E., 2013. Defining taxonomic ranks. In *The prokaryotes* (pp. 229-254). Springer Berlin Heidelberg.

Konstantinidis, K.T. and Tiedje, J.M., 2005. Genomic insights that advance the species definition for prokaryotes. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(7), pp.2567-2572.

Koonin, E.V., 2005. Orthologs, paralogs, and evolutionary genomics. *Annu. Rev. Genet.*, *39*, pp.309-338.

Koval, S.F. and Hynes, S.H., 1991. Effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorus. Journal of bacteriology*, *173*(7), pp.2244-2249.

Koval, S.F., 2006. The search for hunters: culture-dependent and-independent methods for analysis of Bdellovibrio and like organisms. In *Predatory Prokaryotes* (pp. 191-211). Springer Berlin Heidelberg.

Koval, S.F., Hynes, S.H., Flannagan, R.S., Pasternak, Z., Davidov, Y. and Jurkevitch, E., 2013. *Bdellovibrio exovorus* sp. nov., a novel predator of *Caulobacter crescentus*. *International journal of systematic and evolutionary microbiology*, *63*(1), pp.146-151.

Koval, S.F., Williams, H.N. and Stine, O.C., 2015. Reclassification of *Bacteriovorax marinus* as *Halobacteriovorax marinus* gen. nov., comb. nov. and *Bacteriovorax litoralis* as *Halobacteriovorax litoralis* comb. nov.; description of Halobacteriovoraceae fam. nov. in the class Deltaproteobacteria. *International journal of systematic and evolutionary microbiology*, *65*(2), pp.593-597.

Kramer T, Westergaard J., 1977. Antigenicity of bdellovibrios. Appl Environ Microbiol 33:967–970.

Laffineur, K., Avesani, V., Cornu, G., Charlier, J., Janssens, M., Wauters, G. and Delmée, M., 2003. Bacteremia Due to a Novel Microbacterium Species in a Patient with Leukemia and Description of Microbacterium paraoxydans sp. nov. *Journal of clinical microbiology*, *41*(5), pp.2242-2246.

Lambert, C. and Sockett, R.E., 2008. Laboratory maintenance of *Bdellovibrio*. *Current protocols in microbiology*, pp.7B-2.

Lambert, C., Cadby, I.T., Till, R., Bui, N.K., Lerner, T.R., Hughes, W.S., Lee, D.J., Alderwick, L.J., Vollmer, W., Sockett, E.R. and Lovering, A.L., 2015. Ankyrin-mediated self-protection during cell invasion by the bacterial predator *Bdellovibrio bacteriovorus*. *Nature communications*, *6*. p. 8884

Lambert, C., Chang, C.Y., Capeness, M.J. and Sockett, R.E., 2010. The first bite—profiling the predatosome in the bacterial pathogen *Bdellovibrio*. *PloS one*, *5*(1), p.e8599.

Lambert, C., Evans, K.J., Till, R., Hobley, L., Capeness, M., Rendulic, S., Schuster, S.C., Aizawa, S.I. and Sockett, R.E., 2006. Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*. *Molecular microbiology*, *60*(2), pp.274-286.

Lambina, V.A., Afinogenova, A.V., Romay, P.Z., Konovalova, S.M. and Andreev, L.V., 1982. New species of exoparasitic bacteria of the genus *Micavibrio* infecting gram-positive bacteria. *Mikrobiologiia*, *52*(5), pp.777-780.

Langille, M.G. and Brinkman, F.S., 2009. IslandViewer: an integrated interface for computational identification and visualization of genomic islands. *Bioinformatics*, *25*(5), pp.664-665.

Lebba, V., Santangelo, F., Totino, V., Nicoletti, M., Gagliardi, A., De Biase, R.V., Cucchiara, S., Nencioni, L., Conte, M.P. and Schippa, S., 2013. Higher prevalence and abundance of *Bdellovibrio bacteriovorus* in the human gut of healthy subjects. *PLoS One*, *8*(4), p.e61608.

Lebba, V., Totino, V., Santangelo, F., Gagliardi, A., Ciotoli, L., Virga, A., Ambrosi, C., Pompili, M., De Biase, R.V., Selan, L. and Artini, M., 2014. *Bdellovibrio bacteriovorus* directly attacks *Pseudomonas aeruginosa* and *Staphylococcus aureus* Cystic fibrosis isolates. *Frontiers in microbiology*, *5*:280

Lerner, T.R., Lovering, A.L., Bui, N.K., Uchida, K., Aizawa, S.I., Vollmer, W. and Sockett, R.E., 2012. Specialized peptidoglycan hydrolases sculpt the intra-bacterial niche of predatory *Bdellovibrio* and increase population fitness. *Plos pathogens*, *8*(2), p.e1002524.

Li, H., Liu, C., Chen, L., Zhang, X. and Cai, J., 2011. Biological characterization of two marine Bdellovibrio-and-like organisms isolated from Daya bay of Shenzhen, China and their application in the elimination of *Vibrio parahaemolyticus* in oyster. *International journal of food microbiology*, *151*(1), pp.36-43.

Li, L., Stoeckert, C.J. and Roos, D.S., 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome research*, *13*(9), pp.2178-2189.

Lida, Y., Hobley, L., Lambert, C., Fenton, A.K., Sockett, R.E. and Aizawa, S.I., 2009. Roles of multiple flagellins in flagellar formation and flagellar growth post bdelloplast lysis *in Bdellovibrio bacteriovorus*. *Journal of molecular biology*, *394*(5), pp.1011-1021.

Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., Procházková, K., Adkins, I., Hejnová-Holubová, J., Sadílková, L. and Morová, J., 2010. RTX proteins: a highly diverse family secreted by a common mechanism. *FEMS microbiology reviews*, *34*(6), pp.1076-1112.

Loozen, G., Boon, N., Pauwels, M., Slomka, V., Herrero, E.R., Quirynen, M. and Teughels, W., 2015. Effect of *Bdellovibrio bacteriovorus* HD100 on multispecies oral communities. *Anaerobe*, *35*, pp.45-53.

Mahmoud, K.K. and Koval, S.F., 2010. Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio*. *Microbiology*, *156*(4), pp.1040-1051.

Mahmoud, K.K., McNeely, D., Elwood, C. and Koval, S.F., 2007. Design and performance of a 16S rRNA-targeted oligonucleotide probe for detection of members of the genus *Bdellovibrio* by fluorescence in situ hybridization. *Applied and environmental microbiology*, *73*(22), pp.7488-7493.

Majewski, P., Wieczorek, P., Łapuć, I., Ojdana, D., Sieńko, A., Sacha, P., Kłoczko, J. and Tryniszewska, E., 2017. Emergence of multidrug-resistant *Citrobacter freundii* ST8 harboring unusual VIM-4 gene cassette in Poland. *International Journal of Infectious Diseases*.

Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'brien, S.J., Jones, T.F., Fazil, A., Hoekstra, R.M. and International Collaboration on Enteric Disease "Burden of Illness" Studies, 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases*, *50*(6), pp.882-889.

Manickam, N., Mau, M. and Schlömann, M., 2006. Characterization of the novel HCH-degrading strain, *Microbacterium* sp. ITRC1. *Applied microbiology and biotechnology*, *69*(5), pp.580-588.

Marbach, A., Varon, M. and Shilo, M., 1975. Properties of marine bdellovibrios. *Microbial ecology*, *2*(4), pp.284-295.

Martínez, V., Herencias, C., Jurkevitch, E. and Prieto, M.A., 2016. Engineering a predatory bacterium as a proficient killer agent for intracellular bio-products recovery: The case of the polyhydroxyalkanoates. *Scientific reports*, *6*, p.24381.

McNeely, D., Chanyi, R.M., Dooley, J.S., Moore, J.E. and Koval, S.F., 2016. Biocontrol of *Burkholderia cepacia* complex bacteria and bacterial phytopathogens by *Bdellovibrio bacteriovorus. Canadian Journal of Microbiology*, *63*(4), pp.350-358.

Medina, A.A., Shanks, R.M. and Kadouri, D.E., 2008. Development of a novel system for isolating genes involved in predator-prey interactions using host independent derivatives of Bdellovibrio bacteriovorus 109J. *BMC microbiology*, *8*(1), p.33.

Miyoshi, S.I., 2013. Extracellular proteolytic enzymes produced by human pathogenic Vibrio species. *Frontiers in microbiology*, *4*.

Monnappa, A.K., Bari, W., Choi, S.Y. and Mitchell, R.J., 2016. Investigating the responses of human epithelial cells to predatory bacteria. *Scientific reports*, *6*, p.33485.

Moradigaravand, D., Martin, V., Peacock, S.J. and Parkhill, J., 2017. Evolution and Epidemiology of Multidrug-Resistant *Klebsiella pneumoniae* in the United Kingdom and Ireland. *MBio*, *8*(1), pp.e01976-16.

Moreno-Hagelsieb, G. and Hudy-Yuffa, B., 2014. Estimating overannotation across prokaryotic genomes using BLAST+, UBLAST, LAST and BLAT. *BMC research notes*, *7*(1), p.651.

Muñoz-Dorado, J., Marcos-Torres, F.J., García-Bravo, E., Moraleda-Muñoz, A. and Pérez, J., 2016. Myxobacteria: moving, killing, feeding, and surviving together. *Frontiers in microbiology*, *7*(781), pp. 1-18

Nakamura, M., 1972. Alteration of *Shigella* pathogenicity by other bacteria. *The American journal of clinical nutrition*, *25*(12), pp.1441-1451.

Negus, D., Moore, C., Baker, M., Raghunathan, D., Tyson, J. and Sockett, R.E., 2017. Predator Versus Pathogen: How Does Predatory *Bdellovibrio bacteriovorus* Interface with the Challenges of Killing Gram-Negative Pathogens in a Host Setting?. *Annual Review of Microbiology*, *71*, pp.441-457.

Núñez, M.E., Martin, M.O., Duong, L.K., Ly, E. and Spain, E.M., 2003. Investigations into the life cycle of the bacterial predator *Bdellovibrio bacteriovorus* 109J at an interface by atomic force microscopy. *Biophysical journal*, *84*(5), pp.3379-3388.

Olanya, O.M. and Lakshman, D.K., 2015. Potential of predatory bacteria as biocontrol agents for foodborne and plant pathogens. *Journal of Plant Pathology*, *97 (3),* pp 405-417

Otto, M., 2014. Staphylococcus aureus toxins. *Current opinion in microbiology*, *17*, pp.32-37.

Oyedara, O.O., Luna-Santillana, D., de Jesus, E., Olguin-Rodriguez, O., Guo, X., Mendoza-Villa, M.A., Menchaca-Arredondo, J.L., Elufisan, T.O., Garza-Hernandez, J.A., Garcia Leon, I. and Rodriguez-Perez, M.A., 2016. Isolation of Bdellovibrio sp. from soil samples in Mexico and their potential applications in control of pathogens. *MicrobiologyOpen*, *5*(6), pp.992-1002.

Page, J.A., Lubbers, B., Maher, J., Ritsch, L. and Gragg, S.E., 2015. Investigation into the efficacy of *Bdellovibrio bacteriovorus* as a novel preharvest intervention to control *Escherichia coli* O157: H7 and *Salmonella* in cattle using an in vitro model. *Journal of food protection*, *78*(9), pp.1745-1749.

Pan, A., Chanda, I. and Chakrabarti, J., 2011. Analysis of the genome and proteome composition of Bdellovibrio bacteriovorus: indication for recent prey-derived horizontal gene transfer. *Genomics*, *98*(3), pp.213-222.

Park, J.T. and Mahadevan, S.U.B.R.A.M.O.N.Y., 1988. Penicillin-binding proteins of bdellovibrios. *Journal of bacteriology*, *170*(8), pp.3750-3751.

Parker, C.A. and Grove, P.L., 1970. *Bdellovibrio bacteriovorus* parasitizing rhizobium in Western Australia. *Journal of Applied Microbiology*, *33*(1), pp.253-255.

Prehna, G., Ramirez, B.E. and Lovering, A.L., 2014. The lifestyle switch protein Bd0108 of Bdellovibrio bacteriovorus is an intrinsically disordered protein. PloS one, 9(12), p.e115390.

Reiner, A.M. and Shilo, M., 1969. Host-independent growth of Bdellovibrio bacteriovorus in microbial extracts. Microbiology, 59(3), pp.401-410.

Rendulic, S., Jagtap, P., Rosinus, A., Eppinger, M., Baar, C., Lanz, C., Keller, H., Lambert, C., Evans, K.J., Goesmann, A. and Meyer, F., 2004. A predator unmasked: life cycle of Bdellovibrio bacteriovorus from a genomic perspective. Science, 303(5658), pp.689-692.

Richardson, I.R., 1990. The incidence of *Bdellovibrio* spp. in man-made water systems: coexistence with *legionellas*. *Journal of Applied Microbiology*, *69*(1), pp.134-140.

Rittenberg, S.C. and Shilo, M., 1970. Early host damage in the infection cycle of *Bdellovibrio bacteriovorus*. *Journal of bacteriology*, *102*(1), pp.149-160.

Romanowski, E.G., Stella, N.A., Brothers, K.M., Yates, K.A., Funderburgh, M.L., Funderburgh, J.L., Gupta, S., Dharani, S., Kadouri, D.E. and Shanks, R.M., 2016. Predatory bacteria are nontoxic to the rabbit ocular surface. *Scientific reports*, *6*, p.30987.

Romo, A.J., Ruby, E.G. and Saier, M.H., 1992. Effect of *Bdellovibrio bacteriovirus* infection on the phosphoenolpyruvate: sugar phosphotransferase system in *Escherichia coli*: evidence for activation of cytoplasmic proteolysis. *Research in microbiology*, *143*(1), pp.5-14.

Roschanski, N., Klages, S., Reinhardt, R., Linscheid, M. and Strauch, E., 2011. Identification of genes essential for prey-independent growth of *Bdellovibrio bacteriovorus* HD100. Journal of bacteriology, 193(7), pp.1745-1756.

Ruby, E.G., 1992. The genus *Bdellovibrio*. In *The prokaryotes* (pp. 3400-3415). Springer New York.

Safdar, N., Sengupta, S., Musuuza, J.S., Juthani-Mehta, M., Drees, M., Abbo, L.M., Milstone, A.M., Furuno, J.P., Varman, M., Anderson, D.J. and Morgan, D.J., 2017. Status of the Prevention of Multidrug-Resistant Organisms in International Settings: A Survey of the Society for Healthcare Epidemiology of America Research Network. *Infection Control & Hospital Epidemiology*, *38*(1), pp.53-60.

Sakharkar, K.R., Dhar, P.K. and Chow, V.T., 2004. Genome reduction in prokaryotic obligatory intracellular parasites of humans: a comparative analysis. *International Journal of Systematic and Evolutionary Microbiology*, *54*(6), pp.1937-1941.

Saxon, E.B., Jackson, R.W., Bhumbra, S., Smith, T. and Sockett, R.E., 2014. *Bdellovibrio bacteriovorus* HD100 guards against Pseudomonas tolaasii brown-blotch lesions on the surface of post-harvest Agaricus bisporus supermarket mushrooms. *BMC microbiology*, *14*(1), p.163.

Schelling, M. and Conti, S., 1986. Host receptor sites involved in the attachment of *Bdellovibrio bacteriovorus* and *Bdellovibrio stolpii*. *FEMS microbiology letters*, *36*(2-3), pp.319-323.

Scherff, R.H., 1973. Control of bacterial blight of soybean by. *Bdellovibrio bacteriovorus*, *328*, pp.400-402.

Schoeffield, A.J., Williams, H.N., Turng, B.F. and Falkler, W.A., 1996. A comparison of the survival of intraperiplasmic and attack phase bdellovibrios with reduced oxygen. *Microbial ecology*, *32*(1), pp.35-46.

Schwudke, D., Bernhardt, A., Beck, S., Madela, K., Linscheid, M.W., Appel, B. and Strauch, E., 2005. Transcriptional activity of the host-interaction locus and a putative pilin gene of *Bdellovibrio bacteriovorus* in the predatory life cycle. Current microbiology, 51(5), pp.310-316.

Schwudke, D., Linscheid, M., Strauch, E., Appel, B., Zähringer, U., Moll, H., Müller, M., Brecker, L., Gronow, S. and Lindner, B., 2003. The Obligate Predatory *Bdellovibrio bacteriovorus* Possesses a Neutral Lipid A Containing α-D-Mannoses That Replace Phosphate Residues Similarities and differences between the lipid as and the lipopolysaccharides of the wild type strain *B. bacteriovorus* HD100 and its host-independent derivative HI100. *Journal of Biological Chemistry*, *278*(30), pp.27502-27512.

Schwudke, D., Strauch, E., Krueger, M. and Appel, B., 2001. Taxonomic studies of predatory bdellovibrios based on 16S rRNA analysis, ribotyping and the *hit* locus and characterization of isolates from the gut of animals. *Systematic and applied microbiology*, *24*(3), pp.385-394.

Seidler, R.J. and Starr, M.P., 1969. Factors affecting the intracellular parasitic growth of *Bdellovibrio bacteriovorus* developing within *Escherichia coli*. *Journal of bacteriology*, 97(2), pp.912-923.

Seidler, R.J. and Starr, M.P., 1969. Isolation and characterization of host-independent bdellovibrios. *Journal of bacteriology*, *100*(2), pp.769-785.

Seidler, R.J., Mandel, M. and Baptist, J.N., 1972. Molecular heterogeneity of the bdellovibrios: evidence of two new species. *Journal of bacteriology*, *109*(1), pp.209-217.

Seidler, R.J., Starr, M.P. and Mandel, M., 1969. Deoxyribonucleic acid characterization of bdellovibrios. *Journal of bacteriology*, *100*(2), pp.786-790.

Severin, A. I., Afinogenova, A. V., Erova, T. E., Bobyk, M. A. & Lambina, V. A.,1981. Comparative study of the membrane protein composition of bacteria in the genus Bdellovibrio. Mikrobiologiya 50, pp.980–984 (in Russian).

Shatzkes, K., Singleton, E., Tang, C., Zuena, M., Shukla, S., Gupta, S., Dharani, S., Onyile, O., Rinaggio, J., Connell, N.D. and Kadouri, D.E., 2016. Predatory bacteria attenuate *Klebsiella pneumoniae* burden in rat lungs. *MBio*, *7*(6), pp.e01847-16.

Shatzkes, K., Singleton, E., Tang, C., Zuena, M., Shukla, S., Gupta, S., Dharani, S., Rinaggio, J., Kadouri, D.E. and Connell, N.D., 2017. Examining the efficacy of intravenous administration of predatory bacteria in rats. *Scientific Reports*, *7,* p1864

Shatzkes, K., Tang, C., Singleton, E., Shukla, S., Zuena, M., Gupta, S., Dharani, S., Rinaggio, J., Connell, N.D. and Kadouri, D.E., 2017. Effect of predatory bacteria on the gut bacterial microbiota in rats. *Scientific Reports*, *7*, p.43483.

Shemesh, Y. and Jurkevitch, E., 2004. Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environmental Microbiology*, *6*(1), pp.12-18.

Shilo, M. and Bruff, B., 1965. Lysis of Gram-negative bacteria by host-independent ectoparasitic Bdellovibrio bacteriovorus isolates. Microbiology, 40(3), pp.317-328.

Simpson, F.J. and Robinson, J., 1968. Some energy-producing systems in *Bdellovibrio bacteriovorus*. strain, 6-5-S. *Canadian Journal of Biochemistry*, 1968, 46(8): pp.865-873

Snyder, A.R., Williams, H.N., Baer, M.L., Walker, K.E. and Stine, O.C., 2002. 16S rDNA sequence analysis of environmental *Bdellovibrio*-and-like organisms (BALO) reveals extensive diversity. *International journal of systematic and evolutionary microbiology*, *52*(6), pp.2089-2094.

Sockett, R.E. and Lambert, C., 2004. *Bdellovibrio* as therapeutic agents: a predatory renaissance? *Nature reviews. Microbiology*, *2*(8), p.669.

Sockett, R.E., 2009. Predatory lifestyle of *Bdellovibrio bacteriovorus*. *Annual review of microbiology*, *63*, pp.523-539.

Song, W.Y., 2004. Identification and characterization of. *Bdellovibrio bacteriovorus***,** a predator of *Burkholderia glumae*. *Journal of microbiology and biotechnology*, 14(1), pp 48-55.

Starr, M.P. and Baigent, N.L., 1966. Parasitic interaction of Bdellovibrio bacteriovorus with other bacteria. *Journal of bacteriology*, *91*(5), pp.2006-2017.

Starr, M.P. and Stolp, H., 1976. Chapter VI *Bdellovibrio* Methodology. *Methods in microbiology*, *9*, pp.217-244.

Starr, M.P., Stolp, H., Trüper, H.G., Balows, A. and Schlegel, H.G. eds., 2013. *The prokaryotes: a handbook on habitats, isolation and identification of bacteria*. Springer Science & Business Media.

Stolp, H. and Petzold, H., 1962. Untersuchungen über einen obligat parasitischen Mikroorganismus mit lytischer Aktivität für Pseudomonas-Bakterien. Journal of Phytopathology, 45(4), pp.364-390.

Stolp, H. and Starr, M.P., 1963. Bdellovibrio bacteriovorus gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. Antonie Van Leeuwenhoek, 29(1), pp.217-248.

Strauch, E., Schwudke, D. and Linscheid, M., 2007. Predatory mechanisms of *Bdellovibrio* and like organisms. *Future Microbiology*, 2(1), pp. 63-67.

Sun, Y., Ye, J., Hou, Y., Chen, H., Cao, J. and Zhou, T., 2017. Predation efficacy of *Bdellovibrio bacteriovorus* on multidrug-resistant clinical pathogens and their corresponding biofilms. *Japanese Journal of Infectious Diseases*, pp.JJID-2016.

Sutton, D.C. and Besant, P.J., 1994. Ecology and characteristics of bdellovibrios from three tropical marine habitats. *Marine Biology*, *119*(2), pp.313-320.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), pp.2725-2729.

Tan, T.Y., Tan, J.S.M., Tay, H., Chua, G.H., Ng, L.S.Y. and Syahidah, N., 2013. Multidrug-resistant organisms in a routine ward environment: differential propensity for environmental dissemination and implications for infection control. *Journal of medical microbiology*, *62*(5), pp.766-772.

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E.P., Zaslavsky, L., Lomsadze, A., Pruitt, K.D., Borodovsky, M. and Ostell, J., 2016. NCBI prokaryotic genome annotation pipeline. *Nucleic acids research*, *44*(14), pp.6614-6624.

Taylor, V.I., Baumann, P., Reichelt, J.L. and Allen, R.D., 1974. Isolation, enumeration, and host range of marine bdellovibrios. *Archives of microbiology*, *98*(1), pp.101-114.

Thomashow, M.F. and Rittenberg, S.C., 1978. Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: solubilization of Escherichia coli peptidoglycan. *Journal of bacteriology*, *135*(3), pp.998-1007.

Thomashow, M.F. and Rittenberg, S.C., 1978. Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: N-deacetylation of Escherichia coli peptidoglycan amino sugars. *Journal of bacteriology*, *135*(3), pp.1008-1014.

Thomashow, M.F. and Rittenberg, S.C., 1978. Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: attachment of long-chain fatty acids to escherichia coli peptidoglycan. *Journal of bacteriology*, *135*(3), pp.1015-1023.

Thompson, C.C., Chimetto, L., Edwards, R.A., Swings, J., Stackebrandt, E. and Thompson, F.L., 2013. Microbial genomic taxonomy. *BMC genomics*, *14*(1), p.913.

Thys, R.C.S., Lucas, F.S., Riffel, A., Heeb, P. and Brandelli, A., 2004. Characterization of a protease of a feather-degrading Microbacterium species. *Letters in Applied Microbiology*, *39*(2), pp.181-186.

Toft, C. and Andersson, S.G., 2010. Evolutionary microbial genomics: insights into bacterial host adaptation. *Nature reviews. Genetics*, *11*(7), p.465.

Torrella, F., Guerrero, R. and Seidler, R.J., 1978. Further taxonomic characterization of the genus *Bdellovibrio*. *Canadian journal of microbiology*, *24*(11), pp.1387-1394.

Tsai, I.J., Otto, T.D. and Berriman, M., 2010. Improving draft assemblies by iterative mapping and assembly of short reads to eliminate gaps. *Genome biology*, *11*(4), p.R41.

Tudor, J.J. and McCann, M.P., 2006. Genomic analysis and molecular biology of predatory prokaryotes. In *Predatory Prokaryotes* (pp. 153-189). Springer Berlin Heidelberg.

Tudor, J.J., Davis, J.J., Panichella, M. and Zwolak, A., 2008. Isolation of predation-deficient mutants of Bdellovibrio bacteriovorus by using transposon mutagenesis. Applied and environmental microbiology, 74(17), pp.5436-5443.

Tudor, J.J., McCann, M.P. and Acrich, I.A., 1990. A new model for the penetration of prey cells by bdellovibrios. *Journal of bacteriology*, *172*(5), pp.2421-2426.

Uematsu, T., 1980. Ecology of Bdellovibrio parasitic to rice bacterial leaf blight pathogen, *Xanthomonas oryzae*. *Review of Plant Protection Research*, *13*, pp.12-26.

Van Essche, M., Quirynen, M., Sliepen, I., Loozen, G., Boon, N., Van Eldere, J. and Teughels, W., 2011. Killing of anaerobic pathogens by predatory bacteria. *Molecular oral microbiology*, *26*(1), pp.52-61.

Van Essche, M., Quirynen, M., Sliepen, I., Van Eldere, J. and Teughels, W., 2009. *Bdellovibrio bacteriovorus* attacks *Aggregatibacter actinomycetemcomitans*. *Journal of dental research*, *88*(2), pp.182-186.

Van Essche, M., Sliepen, I., Loozen, G., Van Eldere, J., Quirynen, M., Davidov, Y., Jurkevitch, E., Boon, N. and Teughels, W., 2009. Development and performance of a quantitative PCR for the enumeration of Bdellovibrionaceae. *Environmental microbiology reports*, *1*(4), pp.228-233.

Varon, M. and Seijffers, J., 1975. Symbiosis-independent and symbiosis-incompetent mutants of Bdellovibrio bacteriovorus 109J. *Journal of bacteriology*, 124(3), pp.1191-1197

Varon, M. and Shilo, M., 1969. Attachment of *Bdellovibrio bacteriovorus* to cell wall mutants of *Salmonella* spp. and Escherichia coli. *Journal of bacteriology*, *97*(2), p.977.

Venosa, A.D., 1975. Lysis of *Sphaerotilus natans* swarm cells by *Bdellovibrio bacteriovorus*. *Applied microbiology*, *29*(5), pp.702-705.

Vernikos, G.S. and Parkhill, J., 2008. Resolving the structural features of genomic islands: a machine learning approach. *Genome research*, *18*(2), pp.331-342.

Waack, S., Keller, O., Asper, R., Brodag, T., Damm, C., Fricke, W.F., Surovcik, K., Meinicke, P. and Merkl, R., 2006. Score-based prediction of genomic islands in prokaryotic genomes using hidden Markov models. *BMC bioinformatics*, *7*(1), p.142.

Wang, Y., Coleman-Derr, D., Chen, G. and Gu, Y.Q., 2015. OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. *Nucleic acids research*, *43*(W1), pp.W78-W84.

Watanabe, Y., Nakajima, M., Hoshino, T., Jayasimhulu, K., Brooks, E.E. and Kaneshiro, E.S., 2001. A novel sphingophosphonolipid head group 1-hydroxy-2-aminoethyl phosphonate in *Bdellovibrio stolpii*. *Lipids*, *36*(5), pp.513-519.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, *173*(2), pp.697-703.

Williams, H.N., Falkler, W.A. and Shay, D.E., 1980. Incidence of marine bdellovibrios lytic against Vibrio parahaemolyticus in Chesapeake Bay. *Applied and environmental microbiology*, *40*(5), pp.970-972.

Williams, H.N., Schoeffield, A.J., Guether, D., Kelley, J., Shah, D. and Falkler, W.A., 1995. Recovery of bdellovibrios from submerged surfaces and other aquatic habitats. *Microbial ecology*, *29*(1), pp.39-48.

Willis, A.R., Moore, C., Mazon-Moya, M., Krokowski, S., Lambert, C., Till, R., Mostowy, S. and Sockett, R.E., 2016. Injections of predatory bacteria work alongside host immune cells to treat *Shigella* infection in zebrafish larvae. *Current Biology*, *26*(24), pp.3343-3351.

Woese, C.R., 1987. Bacterial evolution. *Microbiological reviews*, *51*(2), p.221.

Yılmaz, H., Çelik, M.A., Şengezer, Ç. and Özkan, M., Use of *Bdellovibrio bacteriovorus* as Biological Cleaning Method for MBRSystems. 2nd International Conference on Emerging Trends in Engineering and Technology (ICETET'2014), May 30-31, 2014 London (UK)

Yu, R., Zhang, S., Chen, Z. and Li, C., 2017. Isolation and application of predatory *Bdellovibrio*-and-like organisms for municipal waste sludge biolysis and dewaterability enhancement. *Frontiers of Environmental Science & Engineering*, *11*(1), p.10.

Zheng, G., Wang, C., Williams, H.N. and Pineiro, S.A., 2008. Development and evaluation of a quantitative real-time PCR assay for the detection of saltwater *Bacteriovorax. Environmental microbiology*, *10*(10), pp.2515-2526.

APPENDICES

APPENDIX 1: Result obtained from the PathogenFinder online software showing *B. bacteriovorus* SSB218315 as non human pathogen.

22/02/2017 PathogenFinder - Results

Center for Genomic Epidemiology

Home Services Instructions Output

The input organism was predicted as non human pathogen

Probability of being a human pathogen 0.477
Input proteome coverage (%) 0.08
Matched Pathogenic Families 1
Matched Not Pathogenic Families 2

Sequences 3597
Total bpp 1163482
Longest seq 2829
Shortest seq 30
Avg seq lenght 323.0

Input Sequence Scaffold_1_SSB18315_2283 # 2409084 # 2409710 # 1 # ID=1_2283;partial=00;start_type=TTG;rbs_motif=None;rbs_spacer=None;gc_cont=0.466

	PROJECT ID	ACCESSION ID	ORGANISMS	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	39579	FN650140	Legionella longbeachae NSW150, complete genome.	Gammaproteobacteria	putative transcriptional regulator (LysR family)	CBJ12615	77.4

Input Sequence Scaffold_1_SSB18315_812 # 843679 # 843813 # -1 # ID=1_812;partial=00;start_type=ATG;rbs_motif=GGAG/GAGG;rbs_spacer=5-10bp;gc_cont=0.378

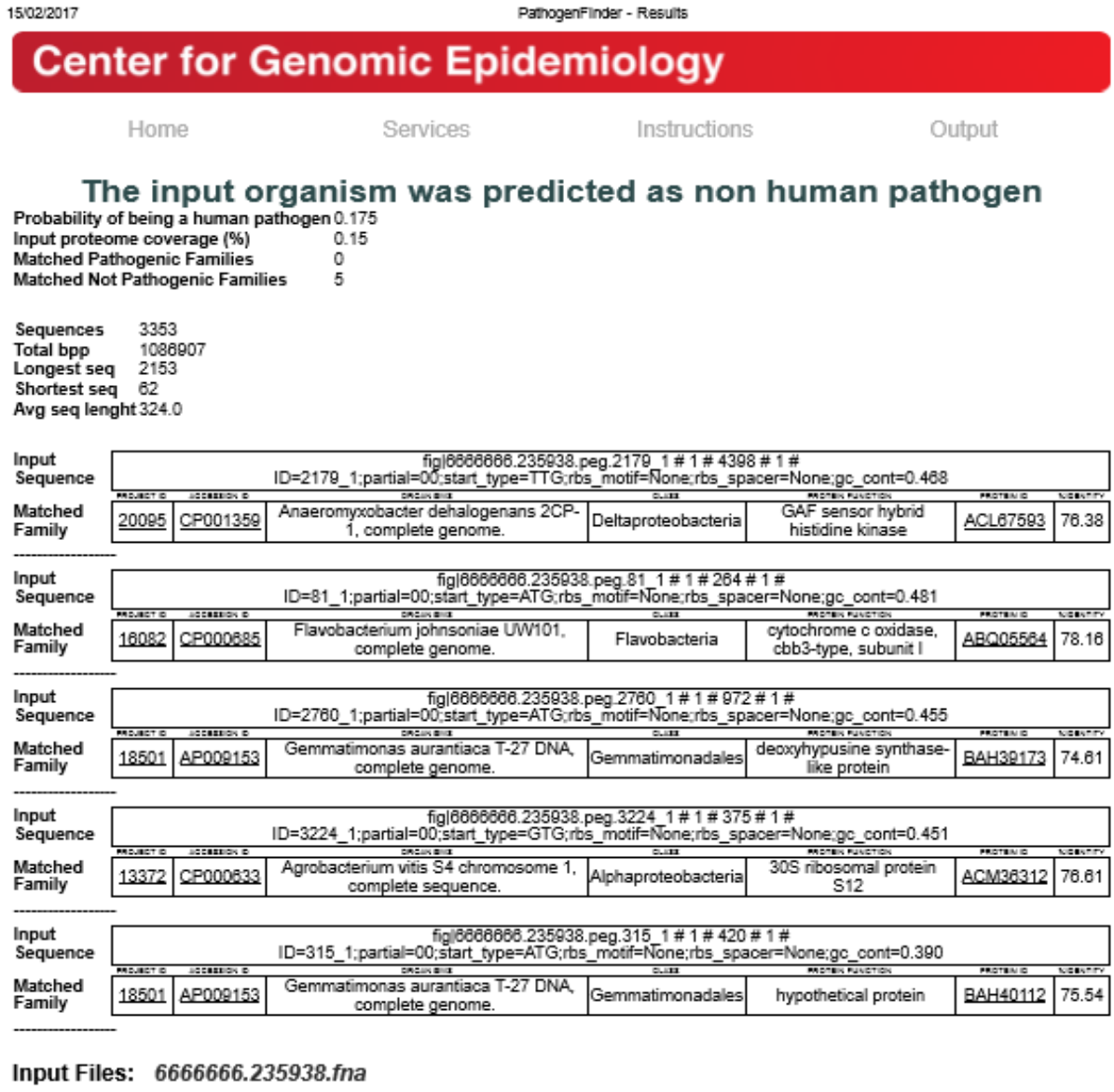
	PROJECT ID	ACCESSION ID	ORGANISMS	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	15771	CP000360	Candidatus Koribacter versatilis Ellin345, complete genome.	Candidatus Koribacter	hypothetical protein	ABF39553	81.82

Input Sequence Scaffold_1_SSB18315_128 # 137943 # 138074 # -1 # ID=1_128;partial=00;start_type=ATG;rbs_motif=AGGAG;rbs_spacer=5-10bp;gc_cont=0.303

	PROJECT ID	ACCESSION ID	ORGANISMS	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	29411	CP001744	Planctomyces limnophilus DSM 3776, complete genome.	Planctomycetacia	histone protein	ADG69442	81.4

Input Files: ***Scaffold_1_SSB18315.fasta***

APPENDIX 2: Result obtained from the PathogenFinder online software showing *Bdellovibrio* sp. SKB1291214 as non human pathogen.



15/02/2017 PathogenFinder - Results

Center for Genomic Epidemiology

Home Services Instructions Output

The input organism was predicted as non human pathogen

Probability of being a human pathogen	0.175
Input proteome coverage (%)	0.15
Matched Pathogenic Families	0
Matched Not Pathogenic Families	5

Sequences	3353
Total bpp	1086907
Longest seq	2153
Shortest seq	62
Avg seq lenght	324.0

Input Sequence	fig|6666666.235938.peg.2179_1 # 1 # 4398 # 1 # ID=2179_1;partial=00;start_type=TTG;rbs_motif=None;rbs_spacer=None;gc_cont=0.468						
	PROJECT ID	ACCESSION ID	ORGANISM	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	20095	CP001359	Anaeromyxobacter dehalogenans 2CP-1, complete genome.	Deltaproteobacteria	GAF sensor hybrid histidine kinase	ACL67593	76.38

Input Sequence	fig|6666666.235938.peg.81_1 # 1 # 264 # 1 # ID=81_1;partial=00;start_type=ATG;rbs_motif=None;rbs_spacer=None;gc_cont=0.481						
	PROJECT ID	ACCESSION ID	ORGANISM	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	16082	CP000685	Flavobacterium johnsoniae UW101, complete genome.	Flavobacteria	cytochrome c oxidase, cbb3-type, subunit I	ABQ05564	78.16

Input Sequence	fig|6666666.235938.peg.2760_1 # 1 # 972 # 1 # ID=2760_1;partial=00;start_type=ATG;rbs_motif=None;rbs_spacer=None;gc_cont=0.455						
	PROJECT ID	ACCESSION ID	ORGANISM	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	18501	AP009153	Gemmatimonas aurantiaca T-27 DNA, complete genome.	Gemmatimonadales	deoxyhypusine synthase-like protein	BAH39173	74.61

Input Sequence	fig|6666666.235938.peg.3224_1 # 1 # 375 # 1 # ID=3224_1;partial=00;start_type=GTG;rbs_motif=None;rbs_spacer=None;gc_cont=0.451						
	PROJECT ID	ACCESSION ID	ORGANISM	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	13372	CP000633	Agrobacterium vitis S4 chromosome 1, complete sequence.	Alphaproteobacteria	30S ribosomal protein S12	ACM36312	76.61

Input Sequence	fig|6666666.235938.peg.315_1 # 1 # 420 # 1 # ID=315_1;partial=00;start_type=ATG;rbs_motif=None;rbs_spacer=None;gc_cont=0.390						
	PROJECT ID	ACCESSION ID	ORGANISM	CLASS	PROTEIN FUNCTION	PROTEIN ID	%IDENTITY
Matched Family	18501	AP009153	Gemmatimonas aurantiaca T-27 DNA, complete genome.	Gemmatimonadales	hypothetical protein	BAH40112	75.54

Input Files: *6666666.235938.fna*

APPENDIX 3. Genomic Islands predicted from the genome of *B. bacteriovorus* SSB218315

Island start	Island end	Length	Method	Gene name	Gene ID	Locus	Gene start	Gene end	Strand	Product
614287	618923	4636	Predicted by at least one method	WP_088566721.1		B9G79_RS00005	1	3769537	-1	hypothetical protein
614287	618923	4636	Predicted by at least one method			B9G79_RS03080	614287	618923	-1	peptidase S74
1082291	1090906	8615	Predicted by at least one method	WP_088566721.1		B9G79_RS00005	1	3769537	-1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564606.1		B9G79_RS05280	1082291	1082785	1	TetR family transcriptional regulator
1082291	1090906	8615	Predicted by at least one method	WP_088564607.1		B9G79_RS05285	1082862	1083980	-1	patatin
1082291	1090906	8615	Predicted by at least one method	WP_088564608.1		B9G79_RS05290	1083994	1084818	-1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564609.1		B9G79_RS05295	1085328	1085600	1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564610.1		B9G79_RS05300	1085604	1085816	1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564611.1		B9G79_RS05305	1086002	1086274	1	transcriptional regulator
1082291	1090906	8615	Predicted by at least one method	WP_088564612.1		B9G79_RS05310	1086545	1086790	1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564613.1		B9G79_RS05315	1086802	1087206	1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564614.1		B9G79_RS05320	1087241	1087687	-1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564615.1		B9G79_RS05325	1087655	1088845	1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564616.1		B9G79_RS05330	1088902	1089375	-1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_088564617.1		B9G79_RS05335	1089750	1089965	1	hypothetical protein
1082291	1090906	8615	Predicted by at least one method	WP_011165187.1		B9G79_RS05345	1090580	1090906	-1	septation protein spoVG
2172097	2177707	5610	Predicted by at least one method	WP_088566721.1		B9G79_RS00005	1	3769537	-1	hypothetical protein
2172097	2177707	5610	Predicted by at least one method	WP_088565467.1		B9G79_RS10495	2172097	2172690	1	hypothetical protein
2172097	2177707	5610	Predicted by at least one method	WP_088566854.1		B9G79_RS10500	2172732	2173292	1	lipid carrier--UDP-N-acetylgalactosaminyltransferase
2172097	2177707	5610	Predicted by at least one method	WP_088565468.1		B9G79_RS10505	2173292	2174161	1	UDP-N-acetylenolpyruvoylglucosamine reductase
2172097	2177707	5610	Predicted by at least one method	WP_088565469.1		B9G79_RS10510	2174706	2176013	1	hypothetical protein
2172097	2177707	5610	Predicted by at least one method	WP_088565470.1		B9G79_RS10515	2176017	2177252	1	hypothetical protein
2172097	2177707	5610	Predicted by at least one method	WP_088565471.1		B9G79_RS10520	2177228	2177707	-1	hypothetical protein

APPENDIX 4. Genomic Islands predicted from the genome of *Bdellovibrio* sp. SKB1291214

Island start	Island end	Length	Method	Gene name	Gene ID	Locus	Gene start	Gene end	Strand	Product
1851606	1862501	10895	Predicted by at least one method	WP_088615526.1	fliS	B9G69_RS09085	1851606	1852010	-1	flagellar export chaperone FliS
1851606	1862501	10895	Predicted by at least one method	WP_088615527.1		B9G69_RS09090	1852061	1853431	-1	flagellar hook associated protein
1851606	1862501	10895	Predicted by at least one method	WP_088615528.1		B9G69_RS09095	1853641	1854012	-1	hypothetical protein
1851606	1862501	10895	Predicted by at least one method	WP_088615529.1		B9G69_RS09100	1854170	1855003	-1	flagellin FliC
1851606	1862501	10895	Predicted by at least one method	WP_088615530.1		B9G69_RS09105	1855787	1856341	1	hypothetical protein
1851606	1862501	10895	Predicted by at least one method	WP_088615531.1		B9G69_RS09110	1856826	1857653	-1	flagellin FliC
1851606	1862501	10895	Predicted by at least one method	WP_088617175.1		B9G69_RS09115	1858128	1858859	-1	murein transglycosylase
1851606	1862501	10895	Predicted by at least one method	WP_088615532.1		B9G69_RS09120	1859150	1861852	1	hybrid sensor histidine kinase/response regulator
1851606	1862501	10895	Predicted by at least one method	WP_088615533.1		B9G69_RS09125	1861860	1862501	-1	thiamine ABC transporter ATP-binding protein
1851606	1862501	10895	Predicted by at least one method	WP_088615534.1		B9G69_RS09130	1862473	1863933	-1	thiamine biosynthesis protein ThiP
1916440	1926734	10294	Predicted by at least one method	WP_088615584.1		B9G69_RS09410	1916440	1917399	1	murein transglycosylase
1916440	1926734	10294	Predicted by at least one method	WP_088615585.1		B9G69_RS09415	1917432	1918619	1	aspartate kinase
1916440	1926734	10294	Predicted by at least one method	WP_088615586.1		B9G69_RS09420	1918742	1919986	-1	hypothetical protein
1916440	1926734	10294	Predicted by at least one method	WP_088615587.1		B9G69_RS09425	1920258	1920743	1	glutathione peroxidase
1916440	1926734	10294	Predicted by at least one method	WP_088615588.1		B9G69_RS09430	1920888	1921214	1	hypothetical protein
1916440	1926734	10294	Predicted by at least one method	WP_088615589.1		B9G69_RS09435	1921373	1921783	1	hypothetical protein
1916440	1926734	10294	Predicted by at least one method	WP_088615590.1		B9G69_RS09440	1921787	1922200	-1	DUF2388 domain-containing protein
1916440	1926734	10294	Predicted by at least one method	WP_088615591.1		B9G69_RS09445	1922542	1923177	1	hypothetical protein
1916440	1926734	10294	Predicted by at least one method	WP_088615592.1		B9G69_RS09450	1923227	1923466	-1	hypothetical protein
1916440	1926734	10294	Predicted by at least one method	WP_088615593.1		B9G69_RS09455	1923618	1924160	1	hypothetical protein
1916440	1926734	10294	Predicted by at least one method	WP_088615594.1	recA	B9G69_RS09460	1924542	1925684	-1	recombinase RecA
1916440	1926734	10294	Predicted by at least one method	WP_088615595.1		B9G69_RS09465	1925766	1926254	-1	CinA family protein
1916440	1926734	10294	Predicted by at least one method	WP_088615596.1		B9G69_RS09470	1926255	1926734	-1	phosphatidylglycerophosphatase A
2370769	2436093	65324	Predicted by at least one method	WP_088615982.1		B9G69_RS11660	2370769	2371140	1	molecular chaperone DnaK
2370769	2436093	65324	Predicted by at least one method	WP_088615983.1		B9G69_RS11665	2371322	2371693	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088615984.1	lon	B9G69_RS11670	2371843	2374248	1	endopeptidase La
2370769	2436093	65324	Predicted by at least one method	WP_088615985.1		B9G69_RS11675	2374432	2374953	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088615986.1		B9G69_RS11680	2374886	2375740	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088617218.1		B9G69_RS11685	2375816	2376241	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088615987.1		B9G69_RS11690	2376568	2377773	1	hypothetical protein

APPENDIX 4. Genomic Islands predicted from the genome of *Bdellovibrio* sp. SKB1291214 (Continued)

Island start	Island end	Length	Method	Gene name	Gene ID	Locus	Gene start	Gene end	Strand	Product
2370769	2436093	65324	Predicted by at least one method	WP_088615988.1		B9G69_RS11695	2377982	2379442	-1	NADH-quinone oxidoreductase subunit N
2370769	2436093	65324	Predicted by at least one method	WP_088615989.1		B9G69_RS11700	2379442	2381034	-1	Fe-S-binding domain-containing protein
2370769	2436093	65324	Predicted by at least one method	WP_088615990.1		B9G69_RS11705	2381038	2382969	-1	NADH-quinone oxidoreductase subunit L
2370769	2436093	65324	Predicted by at least one method	WP_088615991.1		B9G69_RS11710	2382975	2383298	-1	NADH-quinone oxidoreductase subunit NuoK
2370769	2436093	65324	Predicted by at least one method	WP_088615992.1		B9G69_RS11715	2383288	2383824	-1	NADH-quinone oxidoreductase subunit J
2370769	2436093	65324	Predicted by at least one method	WP_088615993.1		B9G69_RS11720	2383835	2384992	-1	NADH-quinone oxidoreductase subunit H
2370769	2436093	65324	Predicted by at least one method	WP_088615994.1		B9G69_RS11725	2384996	2385364	-1	NADH-quinone oxidoreductase subunit A
2370769	2436093	65324	Predicted by at least one method	WP_088615995.1	mfd	B9G69_RS11730	2385484	2389014	1	transcription-repair coupling factor
2370769	2436093	65324	Predicted by at least one method	WP_088615996.1		B9G69_RS11735	2389011	2389805	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088615997.1		B9G69_RS11740	2389863	2391539	1	beta-hexosaminidase
2370769	2436093	65324	Predicted by at least one method	WP_088617219.1		B9G69_RS11745	2391542	2393074	-1	excinuclease ABC subunit C
2370769	2436093	65324	Predicted by at least one method	WP_088615998.1		B9G69_RS11750	2393086	2395503	-1	ATP-dependent helicase
2370769	2436093	65324	Predicted by at least one method	WP_088615999.1		B9G69_RS11755	2395521	2396621	-1	radical SAM protein
2370769	2436093	65324	Predicted by at least one method	WP_088616000.1	atpC	B9G69_RS11760	2396738	2397157	-1	ATP synthase F1 subunit epsilon
2370769	2436093	65324	Predicted by at least one method	WP_088616001.1	atpD	B9G69_RS11765	2397218	2398624	-1	F0F1 ATP synthase subunit beta
2370769	2436093	65324	Predicted by at least one method	WP_088616002.1	atpG	B9G69_RS11770	2398636	2399523	-1	ATP synthase F1 subunit gamma
2370769	2436093	65324	Predicted by at least one method	WP_088616003.1		B9G69_RS11775	2399536	2401059	-1	F0F1 ATP synthase subunit alpha
2370769	2436093	65324	Predicted by at least one method	WP_088616004.1	atpH	B9G69_RS11780	2401062	2401610	-1	ATP synthase F1 subunit delta
2370769	2436093	65324	Predicted by at least one method	WP_088616005.1		B9G69_RS11785	2401607	2402176	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616006.1		B9G69_RS11790	2402191	2402625	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616007.1		B9G69_RS11795	2402739	2403104	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616008.1		B9G69_RS11800	2403095	2404126	-1	chromosome partitioning protein
2370769	2436093	65324	Predicted by at least one method			B9G69_RS11805	2404194	2404949	-1	chromosome partitioning protein
2370769	2436093	65324	Predicted by at least one method			B9G69_RS11810	2405078	2405527	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616009.1	rsmG	B9G69_RS11815	2405667	2406338	-1	16S rRNA (guanine(527)-N(7))-methyltransferase RsmG
2370769	2436093	65324	Predicted by at least one method	WP_088616010.1		B9G69_RS11820	2406325	2408220	-1	tRNA uridine-5-carboxymethylaminomethyl(34) synthesis enzyme MnmG
2370769	2436093	65324	Predicted by at least one method	WP_088616011.1	trmE	B9G69_RS11825	2408224	2409648	-1	tRNA uridine-5-carboxymethylaminomethyl(34) synthesis GTPase MnmE
2370769	2436093	65324	Predicted by at least one method	WP_088616012.1		B9G69_RS11830	2409665	2410228	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616013.1		B9G69_RS11835	2410264	2411904	-1	membrane protein insertase
2370769	2436093	65324	Predicted by at least one method	WP_088617220.1	yidD	B9G69_RS11840	2411882	2412133	-1	membrane protein insertion efficiency factor YidD

APPENDIX 4. Genomic Islands predicted from the genome of *Bdellovibrio* sp. SKB1291214 (Continued)

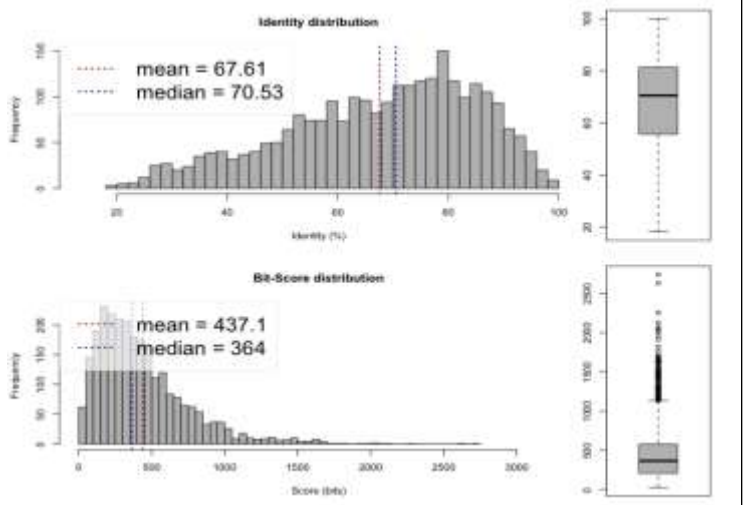
Island start	Island end	Length	Method	Gene name	Gene ID	Locus	Gene start	Gene end	Strand	Product
2370769	2436093	65324	Predicted by at least one method	WP_088616014.1	rnpA	B9G69_RS11845	2412126	2412485	-1	ribonuclease P protein component
2370769	2436093	65324	Predicted by at least one method	WP_088616015.1		B9G69_RS11850	2412475	2412624	-1	50S ribosomal protein L34
2370769	2436093	65324	Predicted by at least one method	WP_088616016.1		B9G69_RS11855	2412840	2414261	1	chromosomal replication initiator protein DnaA
2370769	2436093	65324	Predicted by at least one method	WP_088616017.1	dnaN	B9G69_RS11860	2414497	2415603	1	DNA polymerase III subunit beta
2370769	2436093	65324	Predicted by at least one method	WP_088616018.1		B9G69_RS11865	2415603	2416727	1	DNA replication and repair protein RecF
2370769	2436093	65324	Predicted by at least one method	WP_088616019.1	gyrB	B9G69_RS11870	2416815	2419244	1	DNA topoisomerase (ATP-hydrolyzing) subunit B
2370769	2436093	65324	Predicted by at least one method	WP_088616020.1		B9G69_RS11875	2419268	2421757	1	DNA gyrase subunit A
2370769	2436093	65324	Predicted by at least one method	WP_088616021.1		B9G69_RS11880	2421759	2422556	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616022.1		B9G69_RS11885	2422556	2422780	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616023.1		B9G69_RS11890	2422781	2423152	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616024.1	atpB	B9G69_RS11895	2423127	2423831	1	ATP synthase F0 subunit A
2370769	2436093	65324	Predicted by at least one method	WP_088616025.1		B9G69_RS11900	2423869	2424177	1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616026.1		B9G69_RS11905	2424527	2425894	-1	phosphomannomutase/phosphoglucomutase
2370769	2436093	65324	Predicted by at least one method	WP_088616027.1		B9G69_RS11910	2425944	2426900	-1	pyridoxal phosphate biosynthetic protein
2370769	2436093	65324	Predicted by at least one method	WP_088616028.1		B9G69_RS11915	2426893	2427837	-1	survival protein SurA
2370769	2436093	65324	Predicted by at least one method	WP_088616029.1		B9G69_RS11920	2427830	2428798	-1	parvulin peptidyl-prolyl isomerase
2370769	2436093	65324	Predicted by at least one method	WP_088616030.1		B9G69_RS11925	2428811	2429620	-1	hypothetical protein
2370769	2436093	65324	Predicted by at least one method	WP_088616031.1		B9G69_RS11930	2429674	2430327	-1	DUF374 domain-containing protein
2370769	2436093	65324	Predicted by at least one method	WP_088616032.1		B9G69_RS11935	2430330	2431298	-1	electron transfer flavoprotein subunit alpha/FixB family protein
2370769	2436093	65324	Predicted by at least one method	WP_088616033.1		B9G69_RS11940	2431308	2432081	-1	electron transfer flavoprotein subunit beta
2370769	2436093	65324	Predicted by at least one method	WP_088616034.1		B9G69_RS11945	2432264	2432920	1	cytochrome B subunit
2370769	2436093	65324	Predicted by at least one method	WP_088616035.1	sdhA	B9G69_RS11950	2432931	2434847	1	succinate dehydrogenase flavoprotein subunit
2370769	2436093	65324	Predicted by at least one method	WP_088616036.1		B9G69_RS11955	2434858	2435646	1	succinate dehydrogenase/fumarate reductase iron-sulfur subunit
2370769	2436093	65324	Predicted by at least one method	WP_088616037.1		B9G69_RS11960	2435848	2436093	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088617240.1		B9G69_RS13185	2670900	2671268	1	glyoxalase
2670900	2685361	14461	Predicted by at least one method	WP_088616250.1		B9G69_RS13190	2671270	2672202	-1	response regulator
2670900	2685361	14461	Predicted by at least one method	WP_088616251.1		B9G69_RS13195	2672338	2672883	-1	3'-5' exonuclease domain-containing protein 2
2670900	2685361	14461	Predicted by at least one method	WP_088616252.1		B9G69_RS13200	2672984	2674069	-1	aldo/keto reductase

APPENDIX 4. Genomic Islands predicted from the genome of *Bdellovibrio* sp. SKB1291214 (Continued)

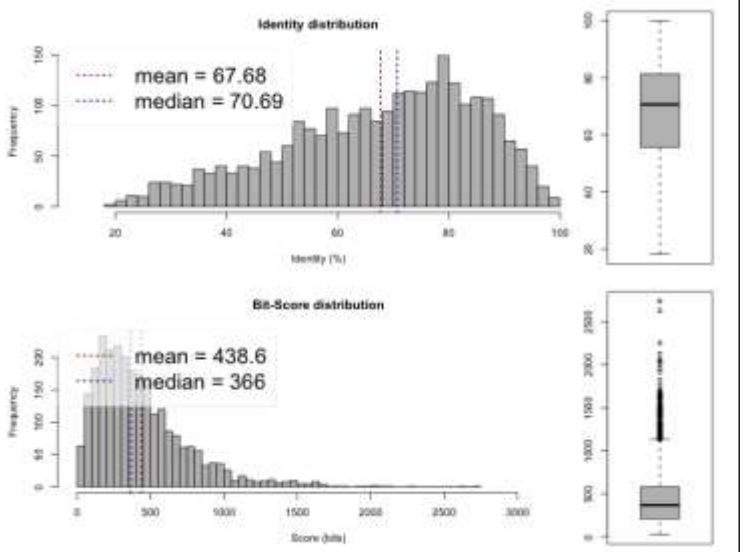
Island start	Island end	Length	Method	Gene name	Gene ID	Locus	Gene start	Gene end	Strand	Product
2670900	2685361	14461	Predicted by at least one method	WP_088616253.1		B9G69_RS13205	2674241	2675188	1	AraC family transcriptional regulator
2670900	2685361	14461	Predicted by at least one method	WP_088616254.1		B9G69_RS13210	2675279	2675677	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616255.1		B9G69_RS13215	2675751	2676146	1	aldehyde-activating protein
2670900	2685361	14461	Predicted by at least one method	WP_088616256.1		B9G69_RS13220	2676357	2677595	1	integrase
2670900	2685361	14461	Predicted by at least one method	WP_088616257.1		B9G69_RS13225	2677609	2678202	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616258.1		B9G69_RS13230	2678289	2678510	1	AlpA family phage regulatory protein
2670900	2685361	14461	Predicted by at least one method	WP_088616259.1		B9G69_RS13235	2678503	2679945	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616260.1		B9G69_RS13240	2680013	2680522	1	terminase small subunit
2670900	2685361	14461	Predicted by at least one method	WP_088616261.1		B9G69_RS13245	2680509	2680760	-1	XRE family transcriptional regulator
2670900	2685361	14461	Predicted by at least one method	WP_088616262.1		B9G69_RS13250	2680818	2681207	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616263.1		B9G69_RS13255	2681250	2681756	-1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616264.1		B9G69_RS13260	2681815	2682003	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616265.1		B9G69_RS13265	2682016	2682954	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616266.1		B9G69_RS13275	2683850	2685361	1	hypothetical protein
2670900	2685361	14461	Predicted by at least one method	WP_088616267.1		B9G69_RS13280	2685358	2686020	-1	glutamine amidotransferase
2675279	2682954	7675	Predicted by at least one method	WP_088616254.1		B9G69_RS13210	2675279	2675677	1	hypothetical protein
2675279	2682954	7675	Predicted by at least one method	WP_088616255.1		B9G69_RS13215	2675751	2676146	1	aldehyde-activating protein
2675279	2682954	7675	Predicted by at least one method	WP_088616256.1		B9G69_RS13220	2676357	2677595	1	integrase
2675279	2682954	7675	Predicted by at least one method	WP_088616257.1		B9G69_RS13225	2677609	2678202	1	hypothetical protein
2675279	2682954	7675	Predicted by at least one method	WP_088616258.1		B9G69_RS13230	2678289	2678510	1	AlpA family phage regulatory protein
2675279	2682954	7675	Predicted by at least one method	WP_088616259.1		B9G69_RS13235	2678503	2679945	1	hypothetical protein
2675279	2682954	7675	Predicted by at least one method	WP_088616260.1		B9G69_RS13240	2680013	2680522	1	terminase small subunit
2675279	2682954	7675	Predicted by at least one method	WP_088616261.1		B9G69_RS13245	2680509	2680760	-1	XRE family transcriptional regulator
2675279	2682954	7675	Predicted by at least one method	WP_088616262.1		B9G69_RS13250	2680818	2681207	1	hypothetical protein
2675279	2682954	7675	Predicted by at least one method	WP_088616263.1		B9G69_RS13255	2681250	2681756	-1	hypothetical protein
2675279	2682954	7675	Predicted by at least one method	WP_088616264.1		B9G69_RS13260	2681815	2682003	1	hypothetical protein
2675279	2682954	7675	Predicted by at least one method	WP_088616265.1		B9G69_RS13265	2682016	2682954	1	hypothetical protein
3571542	3580107	8565	Predicted by at least one method	WP_088613920.1		B9G69_RS00030	3571542	3572150	1	HAD family phosphatase
3571542	3580107	8565	Predicted by at least one method	WP_088613921.1		B9G69_RS00035	3572172	3572597	-1	hypothetical protein
3571542	3580107	8565	Predicted by at least one method	WP_088613922.1		B9G69_RS00040	3572596	3573312	1	hypothetical protein
3571542	3580107	8565	Predicted by at least one method	WP_088613975.1		B9G69_RS00045	3573331	3573624	1	hypothetical protein
3571542	3580107	8565	Predicted by at least one method	WP_088613923.1		B9G69_RS00050	3573737	3574987	1	MFS transporter
3571542	3580107	8565	Predicted by at least one method	WP_088613924.1		B9G69_RS00055	3575039	3575431	1	PaaI family thioesterase
3571542	3580107	8565	Predicted by at least one method	WP_088613925.1		B9G69_RS00065	3575608	3577056	-1	competence protein ComM
3571542	3580107	8565	Predicted by at least one method	WP_088613926.1		B9G69_RS00070	3577141	3578019	-1	tyrosine recombinase XerD
3571542	3580107	8565	Predicted by at least one method	WP_088613927.1		B9G69_RS00075	3578147	3578854	-1	hypothetical protein
3571542	3580107	8565	Predicted by at least one method	WP_088613928.1		B9G69_RS00080	3578964	3579740	-1	hypothetical protein
3571542	3580107	8565	Predicted by at least one method	WP_088613929.1		B9G69_RS00085	3579886	3580107	1	hypothetical protein
3725925	3731598	5673	Predicted by at least one method			B9G69_RS18275	3725925	3726137	-1	hypothetical protein
3725925	3731598	5673	Predicted by at least one method	WP_088617421.1		B9G69_RS18150	3727176	3727994	-1	hypothetical protein
3725925	3731598	5673	Predicted by at least one method	WP_088617422.1		B9G69_RS18155	3728024	3728416	-1	hypothetical protein
3725925	3731598	5673	Predicted by at least one method			B9G69_RS18145	3729495	3730010	-1	hypothetical protein
3725925	3731598	5673	Predicted by at least one method			B9G69_RS17895	3731013	3731598	1	hypothetical protein
3725925	3731598	5673	Predicted by at least one method	WP_088617375.1		B9G69_RS17900	3731593	3734070	-1	hypothetical protein

APPENDIX 5: Amino acid Identity between *Bdellovibrio* sp. SKB1291214 and other reported *Bdellovibrio* strains

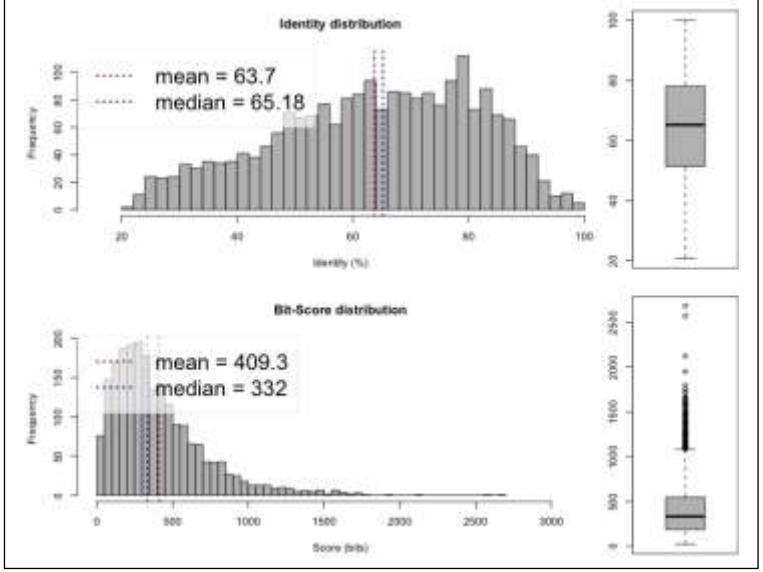
SKB1291214 & HD100



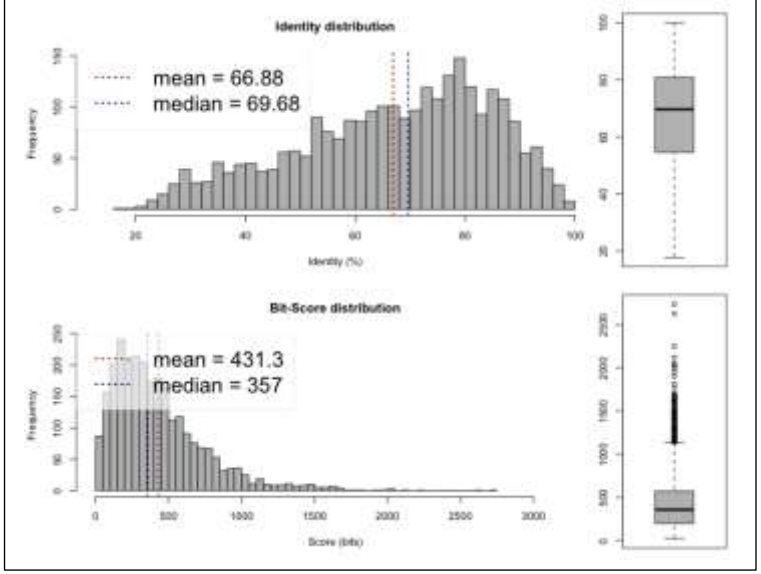
SKB1291214 & 109J



SKB1291214 & W

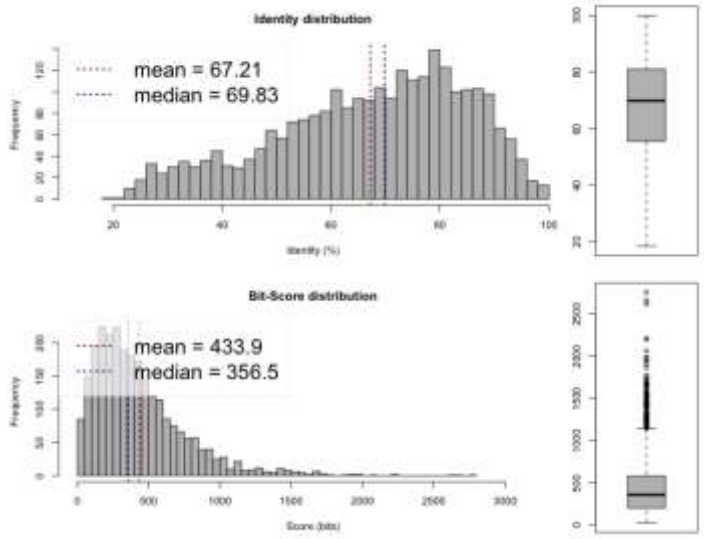


SKB1291214 & TIBERIUS

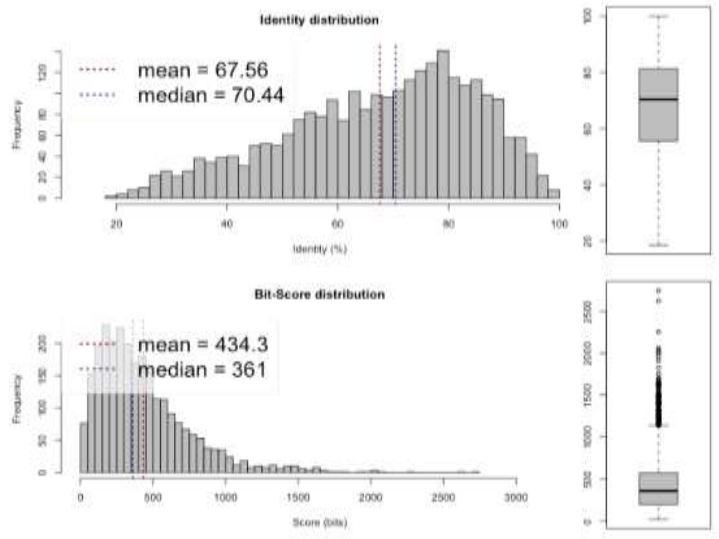


APPENDIX 5: Amino acid Identity between *Bdellovibrio* sp. SKB1291214 and other reported *Bdellovibrio* strains (Continued)

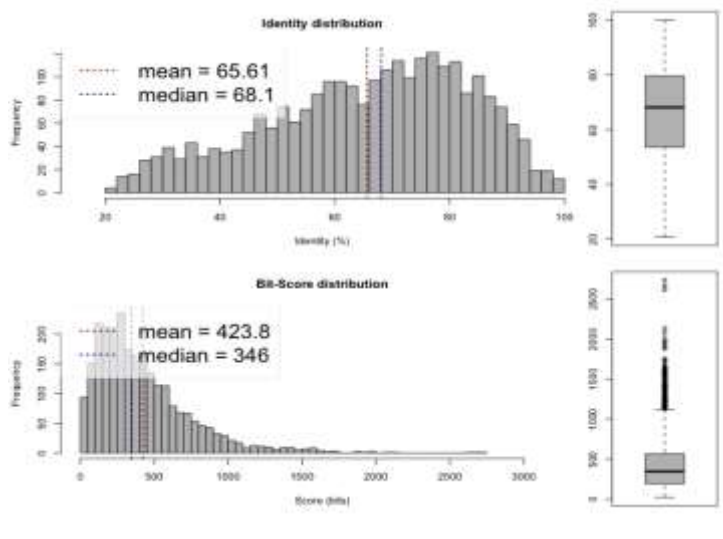
SKB1291214 & EC13



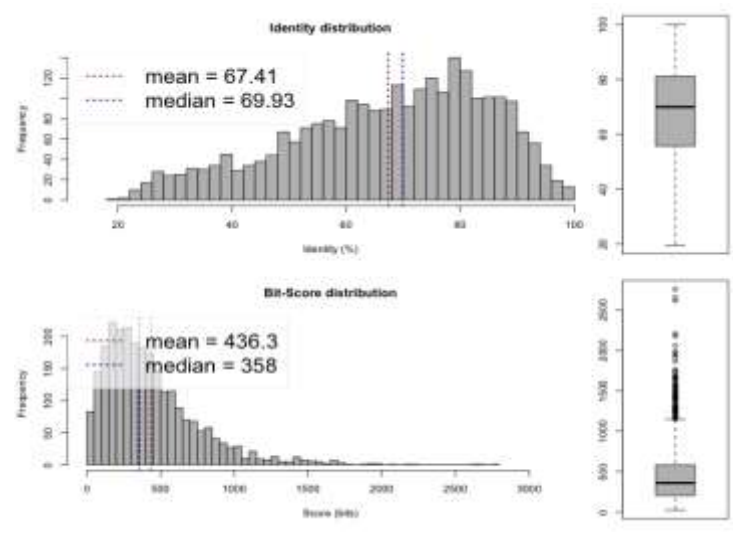
SKB1291214 & SSB218315



SKB1291214 & R0

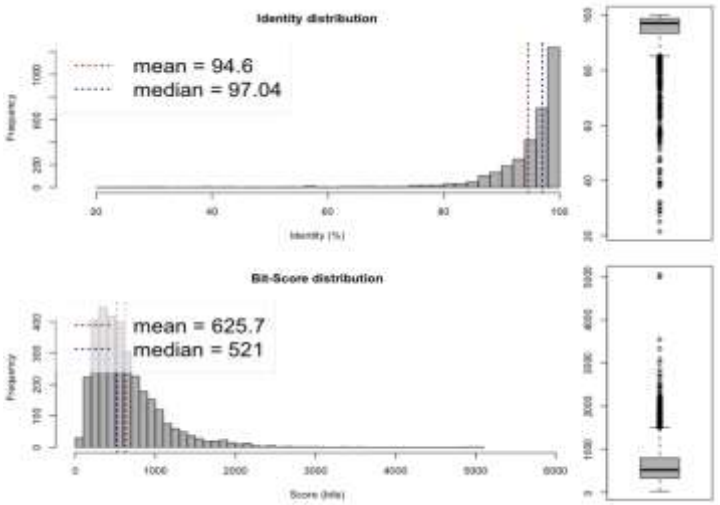


SKB1291214 & BER2

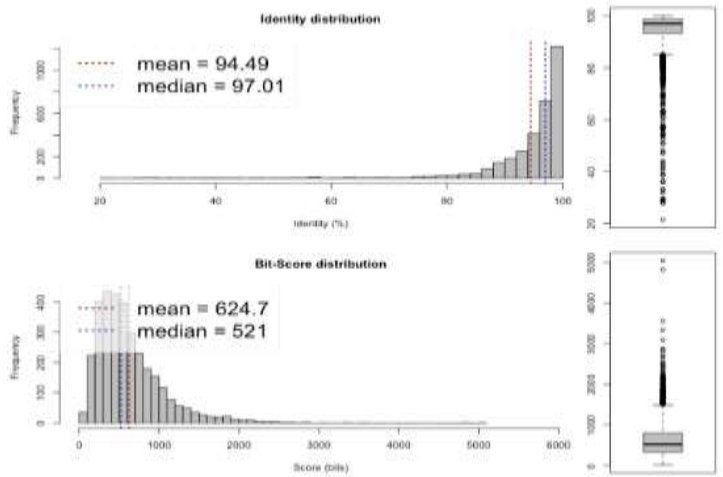


APPENDIX 6: Amino acid Identity between *B bacteriovorus* SSB218315 and other reported *Bdellovibrio* strains

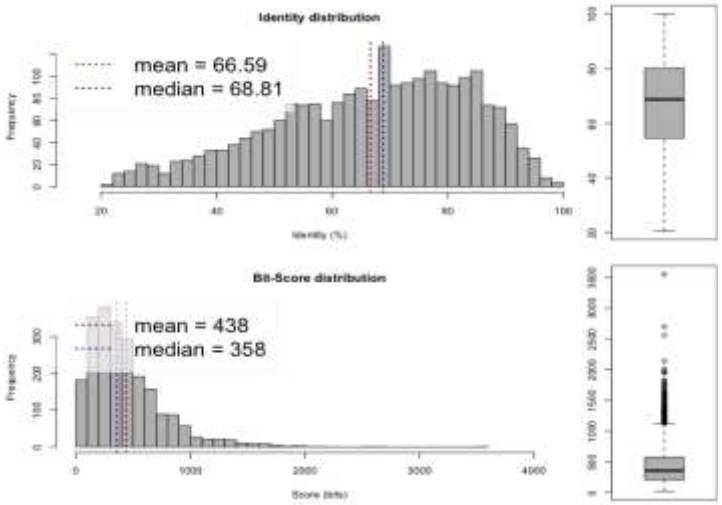
SSB218315 & HD100



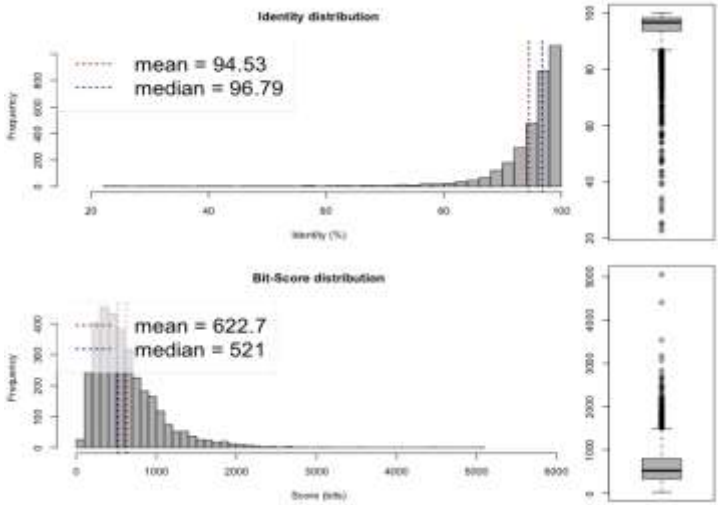
SSB218315 & 109J



SSB218315 & W

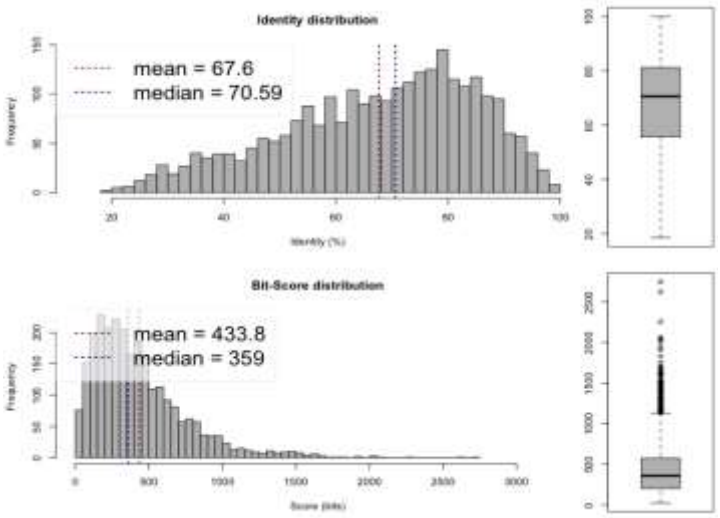


SSB218315 & TIBERIUS

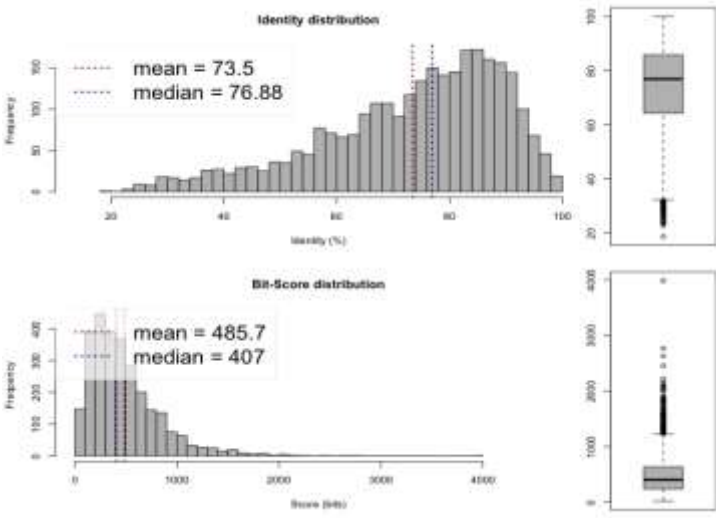


APPENDIX 6: Amino acid Identity between *B bacteriovorus* SSB218315 and other reported *Bdellovibrio* strains (Continued)

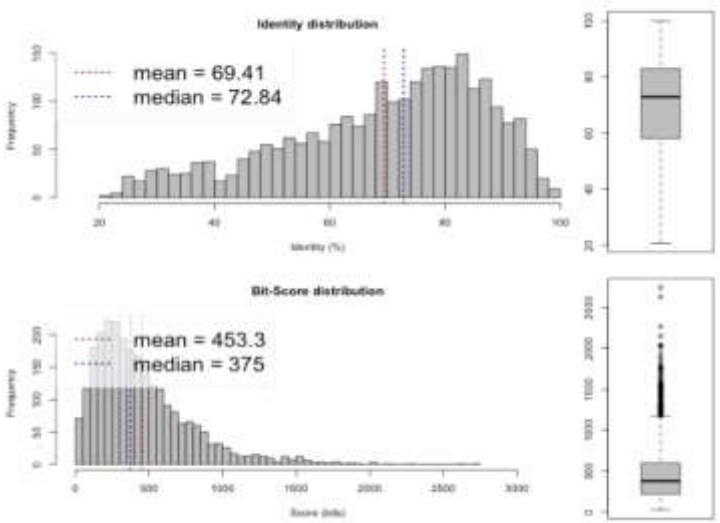
SSB218315 & SKB



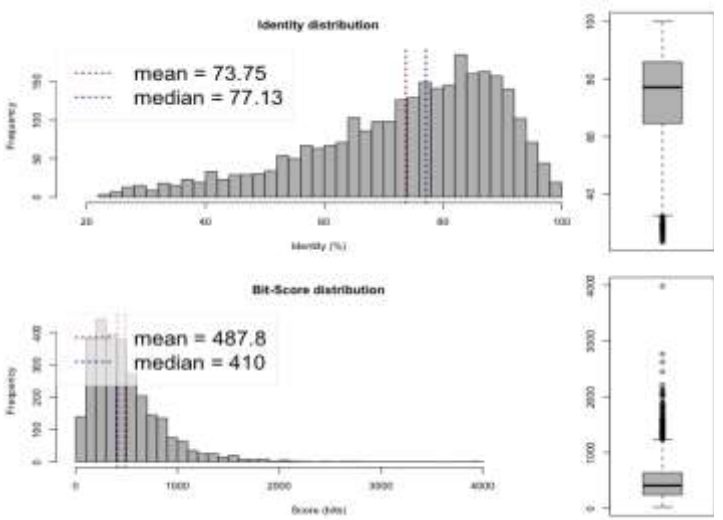
SSB218315 & EC13



SSB218315 & R0



SSB218315 & BER2



APPENDIX 7: MEDIA AND REAGENTS

7.1. Dilute Nutrient Broth (DNB) Medium

0.08 % nutrient broth

3 mM $MgCl_2$. $6H_2O$

2 mM $CaCl_2$. $2H_2O$

0.6 % agar for top agar (Before sterilization, homogenize by heating and dispense 4-ml aliquots in sterile test tubes before the agar solidifies. Then, sterilize. Prior to use, melt in an 80◦C water bath, then maintain at 42◦C to keep molten).

1.9 % for bottom agar (After sterilization, pour into standard 100-mm plates using 20 to 25 ml per plate. Let solidify and dry. Seal in bags and store up to 2 months at 4 ^oC)

Adjust pH with NaOH or HCl [pH 7.2 – 7.4]

Sterilize by autoclaving at 121 ^oC for 15 minutes

7.2. Yeast-Peptone (YP) Medium

10 g/liter peptone

3 g/liter yeast extract

For YP agar, add 10g/liter agar

Adjust pH with NaOH or HCl [pH 6.8 – 7.4]

Sterilize by autoclaving at 121 ^oC for 15 minutes

7.3. HEPES Buffer Medium

25 mM N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES). (SIGMA)

6 ml of 0.5 M $CaCl_2$•2H2O (3 mM final)

3.33 ml of 0.6 M $MgCl_2$.7H2O (2 mM)

Adjust pH with NaOH or HCl [pH 7.2 – 7.4]

Sterilize by autoclaving at 121 ^oC for 15 minutes

7.4. Mueller Hinton Agar (BIOLAB)

Composition g/L

Beef Extract	2.0
Acid Hydrolysate of casein	17.50
Starch	1.50
Agar	17.00
	pH 7.3 ± 0.1 at 25ºC

Preparation: 38 g of MHA was suspended in 1 litre of distilled wáter, brought to boil to dissolve completely and sterilized by autoclaving at 121 ºC for 15 minutes. After cooling to 40 – 45 ºC, the agar was poured into sterile petri dishes and allowed to set.

7.5. Luria Bertani (LB) Agar (Miller)

Enzymatic Digest of Casein	10 g
Yeast Extract	5 g
Sodium Chloride	10 g
Agar	12 g
	pH: 7.3 ± 0.2 at 25°C

Preparation: 37 g of LB agar was suspended in 1 litre of distilled wáter, brought to boil to dissolve completely and sterilized by autoclaving at 121 ºC for 15 minutes. After cooling to 40 – 45 ºC, the agar was poured into sterile petri dishes and allowed to set.

7.6. Luria Bertani (LB) broth (Miller)

Enzymatic Digest of Casein	10 g
Yeast Extract	5 g
Sodium Chloride	10 g
	pH: 7.5 ± 0.2 at 25°C

Preparation: 25 g of LB broth was suspended in 1 litre of distilled wáter, mixed well to dissolve completely, dispensed in test tubes and sterilized by autoclaving at 121 ºC for 15 minutes. After cooling to 40 – 45 ºC, the agar was poured into sterile petri dishes and allowed to set.

7.7. 50 X TAE buffer

Tris base (Sigma)	242.2 g
Glacial acetic acid (Merck)	57.1 ml
EDTA (Sigma)	37.2 g
Distilled water	1 L

7.8. Isopropyl-β-D-thiogalactoside (IPTG)

The IPTG was prepared as a stock of 200 mg/ml and kept as aliquots at –20°C until use

7.9. X-galactosidase (X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

The X-gal was prepared as a stock of 20 mg/ml and kept as aliquots at –20°C until use

Don't forfeit ambition when others may doubt, it's your life to live, you must live it throughout. Learn from your errors don't dwell in the past, never withdraw from a world that is vast. Believe in yourself find the best that is you, let your spirit prevail, steer a course that is true. - Unknown