UNIVERSITI PUTRA MALAYSIA

COMPLETE GENOME SEQUENCING AND ANALYSIS OF Pasteurella multocida STRAIN PMTB2.1 AND EXPRESSION OF SELECTED GENES IN IRON-RESTRICTED ENVIRONMENT

SHAGUFTA JABEEN

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By

SHAGUFTA JABEEN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

December 2017

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DEDICATION

To My Murshid Sayed Abdul Rasheed Mian Sarkar (Rehmatu-Allh Ailah)

To my respectable teachers, specially Prof. Dr. Saleem Hafiz

To my parents and my Husband, my son and to my all dear family members
I dedicate to all, my work with love and gratitude

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

COMPLETE GENOME SEQUENCING AND ANALYSIS OF *Pasteurella multocida* STRAIN *PMTB2.1* AND EXPRESSION OF SELECTED GENES IN IRON-RESTRICTED ENVIRONMENT

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SHAGUFTA JABEEN

December 2017

Chairperman : Abdul Rahman Omar, PhD
Institute : Bioscience

Pasteurella multocida (PM) is a Gram-negative, facultative anaerobic bacterium, belonging to the family *Pasteurellaceae* that commonly found as commensal in the upper respiratory tract of mammals and birds. However, *P. multocida* is often associated with acute as well as chronic infections in avian and bovine leading to significant morbidity and mortality, such as pasteurellosis and hemorrhagic septicemia (HS) in cattle and buffaloes. *P.multocida* subspecies *multocida* strain *PMTB2.1* was first isolated from buffalos died of septicemia. The bacterium has been characterized based on biochemical tests and molecular identification based onPCR. Intrestingly, based on HS causing serogroup B-specific PCR (HSB-PCR), the isolate is not from serogroup B. Hence, an in depth genome wide analysis of *PMTB2.1* was carried out. In this study, the genome of *P. multocida* strain *PMTB2.1* was sequenced using third-generation sequencing technology, PacBio and analysed bioinformatically via *de novo* method followed by in depth characterization of the genome. In addition, expression of selected genes of *PMTB2.1* grown in iron-restricted condition was also demonstrated based on real-time PCR study.

Bioinformatics analysis based on *de novo* assembly of PacBio raw reads generated 3 supercontigs that were assembled to generate a draft genome with unresolved gaps regions. The gaps between the contigs in the assembled draft genome sequence were closed by PCR sequencing with primer walking strategy using Sanger sequencing. Start position of the circular genome of *PMTB2.*1 was set based on homology to reference genome *P. multocida* strain *PM36950* and the circularity of the genome was confirmed by PCR. The complete genome sequence of *P. multocida* strain *PMTB2.1* is composed of a single circular chromosome of 2,315,138 base pairs with 40.32 % GC content and a total of 2,176 potential genes. The genome was submitted to NCBI

under the accession number, CP 007205.1. The annotated complete genome sequences of *PMTB2.1* have 2,097 protein-coding sequences, 19 rRNA genes, 56 tRNA and 4 ncRNA genes. The genome also encode for more than 41 CDS (2%) that involved in iron regulation or iron uptake, 160 virulence genes and 12 antibiotic resistance genes including the complete Tad locus. The tad locus encodes 14 gene including several previously uncharacterized genes such as *flp 2* that play important roles in the adhesion and colonization of the bacteria, biofilm formation as well as in pathogenesis of the disease.

Multi-locus sequence typing against Rural Industries Research and Development Corporation (RIRDC) scheme indicated that *PMTB2.1* matched to alleles from sequence type ST101. Comparative genome analysis showed that *PMTB2.1* is closely related with other *Pasteurella multocida* strains with genomic distance less than 0.13. However, synteny analysis showed that genome structure of *PMTB2.1* is more resembles to that of *P. multocida* serogroup A strain *PM36950* as compared to that of *P. multocida* serogroup F strain *PM70*. However, *PMTB2.1* genome lacks the Integrative Conjugative Element (ICE) of 86 kb that can only be detected in *PM36950*. Nevertheless, two intact prophage sequences of approximately 62 kb that were found in *PMTB2.1*, were absent in *PM36950* and *PM70*. One of the phages is similar to transposable Mu like phage SfMu; however, the phage regions of *PMTB2.1* were not associated with toxin-related genes, as detected in serogroup D toxigenic strain of *P. multocida*. Moreover, *PMTB2.1* complete genome is approximately 34,380 kb smaller than *PM36950* genome (2,349,518 bp), on the other hand approximately 15 kb specific region of *PMTB2.1*was absent in *PM70* genome. The capsular sequence analysis of *PMTB2.1* indicated that it is resembling the capsular sequence of *P. multocida* serogroup A with 99% sequence identity with A:1 capsular sequences. Furthermore, OrthoMCL analysis based on similarity among common genes showed that *PMTB2.1* was clustered with bovine isolates and were separated from other *P. multocida* strains that infect avian and swine.

Since *P. multocida* including *PMTB2.1* has more than 2% of the genome encode for iron-regulated genes, the expression profiling of iron uptake genes namely *fbpb, yfea, fece, fur* and sialidase encoded by *nana* were characterized under iron-restricted environment where *PMTB2.1* was grown in broth with and without iron chelating agent 2,2' Bipyridine. Results of this study reflect that iron-reduced conditions have significant effect on the expression profiles of iron-regulating *gene*s ($p<0.05$) and all of the four iron-related genes (*fbpb, yfea, fece, fur*) behave differently in response to iron reduction in media. The highest relative fold change (281.2 fold) of *fece* gene was observed at early, 30 minutes of treatment reveal that *P. multocida* may utilizes its periplasmic protein at early stage to acquire iron. Furthermore, down-regulation expression of *fece* from 4 to -1.5 with the elevated expression of other genes (*fbpb* and *yfea*) at later time points, 60 and 120 minutes suggest that *PMTB2.1* control their iron requirements in response to iron availability by down regulating the expression of iron proteins. Moreover, the significant increase ($p\leq 0.05$) in *fbpb* expression (25 fold) at time point 60 and in *Yfea* expression (26 fold) at early time point 30 minutes with highest expression (42 fold) at 120 minutes reflect the utilization of multiple iron

systems in *P. multocida* strain *PMTB2.1*. These results demonstrate the importance of iron in the survival of *P. multocida*.

In conclusion, this study has provided insight on the genomic structure of *PMTB2.1* in terms of potential genes that can functions as virulence factors and comparative pathogenomic information of valuable importance for future study in elucidating the mechanisms behind the ability of the bacterium in causing diseases in susceptible animals.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

JUJUKAN GENOM LENGKAP DAN ANALISIS *Pasteurella multocida* STRAIN *PMTB2.1* DAN EKSPRESI GEN TERPILIH DALAM PERSEKITARAN BESI TERHAD

Oleh

SHAGUFTA JABEEN

Disember 2017

Pengerusi : Abdul Rahman Omar, PhD
Institut : Biosains

Pasteurela multocida (PM) ialah bakteria anaerob fakultatif Gram-negatif, kepunyaan famili *Pasteurellaceae* yang biasa ditemui dalam saluran pernafasan atasan mamalia dan unggas. Namun, *P. multocida* selalunya dikaitkan dengan penyakit akut serta kronik dalam ungags dan bovin yang boleh menyebabkan morbiditi dan kadar kematian yang signifikan, seperti penyakit pasteurelosis dan hawar berdarah (HS) dalam lembu dan kerbau. *PMTB2.1* mula diasingkan dari kerbau mati akibat septisemia. Bakteria tersebut telah dicirikan berdasarkan ujian biokimia dan pengenalpastian molekul berdasarkan PCR. Yang menariknya, berdasarkan PCR khusus bagi serokumpulan B penyebab HS (HSB-PCR), isolat tersebut bukan dari serokumpulan B. Maka, analisi genom menyeluruh secara mendalam ke atas *PMTB2.1* telah dilakukan. Dalam kajian ini, genom *P. multocida* strain *PMTB2.1* dijujukkan menggunakan teknologi jujukan generasi ketiga iaitu PacBio dan analisi bioinformatik dilakukan melalui keadah *de novo* dan diikuti oleh pencirian genom secara mendalam. Di samping itu, ekspresi gen terpilih *PMTB2.1* yang ditumbuhkan dalam persekitaran besi terhad juga dicirikan berdasarkan kajian PCR nyata-masa.

Analisis bioinformatik berdasarkan penggabungan data kasar PacBio secara *de novo* menjana tiga "supercontigs" yang dihimpunkan untuk menghasilkan draf genom dimana terdapat bahagian jurang yang tidak dapat dikenalpasti. Penjujukan PCR dengan strategi "primer walking" berdasarkan jujukan Sanger telah digunakan untuk mengenalpasti jurang antara kontig dalam jujukan draf genom. Posisi permulaan genom membulat *PMTB2.1* telah ditetapkan berdasarkan homologi genom rujukan *P. multocida* strain *PM36950* dan bukti genom membulat telah dikenalpasti dengan menggunakan PCR. Jujukan genom lengkap *P. multocida* strain *PMTB2.1* terdiri daripada satu kromosom membulat yang mempunyai 2,315,138 bp dan 40.32 %

jumlah GC serta 2,176 gen potensi. Jujukan genom *PMTB2.1* ini juga telah dimuat naik ke NCBI dengan nombor kemasukan *CP007205.2*. Hasil anotasi genom lengkap mendapati bahawa genom ini mempunyai 2,097 protein pengekodan, 19 gen rRNA, 56 gen tRNA dan 4 gen ncRNA. Genom *PMTB2.1* juga mengekod lebih daripada 41 CDs (2%) yang terlibat di dalam regulasi besi serta pengambilan besi, 160 gen virulen dan 12 gen rintangan antibiotik, termasuk lokus Tad lengkap yang mengekod 14 gene termasuk gene belum terciri seperti *flp2* yang memainkan peranan penting semasa pelekatan dan kolonisasi bakteria, pembentukan biofilm serta pathogenesis penyakit.

Pengelasan jujukan multilokus menggunakan skim *Rural Industries Research and Development Corporation* (RIRDC) menunjukkan *PMTB2.1* sepadan dengan alel daripada jujukan jenis ST101. Analisis komprehensif genom menunjukkan strain *PMTB2.1* adalah berkait rapat dengan *Pasteurella multocida* strain lain dengan jurang genom kurang daripada 0.13. Namun, analisis "synteny" menunjukkan struktur genom *PMTB2.1* adalah lebih bersamaan dengan *P. multocida* serokumpulan A strain *PM36950* berbanding dengan *P. multocida* serokumpulan F strain *PM70*. Tetapi, genom *PMTB2.1* tidak mempunyai "Integrative Conjugative Element" (ICE) yang bersaiz 82 kb yang hanya boleh didapati dalam *PM36950*. Walau bagaimanapun, dua jujukan profaj lengkap bersaiz kira-kira 62kb ditemui hanya di dalam *PMTB2.1*, tetapi tidak didapati dalam *PM36950* dan *PM70*. Salah satu faj didapati serupa dengan faj "transposable" Mu seperti SfMu. Namun, jujukan faj *PMTB2.1* tidak dikaitkan dengan gen toksin, yang terdapat pada *P. multocida* strain toksigenik dlam serokumpulan D. Tambahan pula, genom lengkap *PMTB2.1* adalah kira-kira 34,380 kb lebih kecil daripada genom *PM36950* (2,349,518 bp). Walau bagaimanapun, *PMTB2.1* mempunyai 15 kb bahagian khusus yang tidak hadir dalam genom *PM70*. Analisis jujukan kapsul *PMTB2.1* menunjukkan ia bersamaan dengan jujukan kapsul serokumpulan A *P. multocida* dengan 99% identiti jujukan kapsul A:1. Tambahan pula, analisis OrthoMCL berdasarkan persamaan antara gen umum menunjukan *PMTB2.1* berkumpulan dengan isolat bovin dan adalah diasingkan dari strain *P. multocida* lain yang menjangkiti unggas dan babi.

Bakteria *P. multocida* termasuk strain *PMTB2.1* mempunyai lebih daripada 2% genom yang dikod untuk gen regulasi besi. Profil ekspresi pengambilan besi terutamanya *fbpb, yfea, fece, fur* dan sialidase yang terkod *nana* telah dicirikan di dalam persekitaran besi terhad di mana strain *PMTB2.1* telah ditumbuh di dalam media yang mempunyai pengikat besi, *2, 2' Bipyridine*. Hasil kajian menunjukkan persekitaran besi terhad mempunyai kesan signifikan kepada profil ekspresi gen regulasi besi ($p<0.05$) dan kesemua empat gen (*fbpb, yfea, fece, fur*) bertindak secara berlainan dengan pengurangan besi di dalam media. Ekspresi tertinggi (281.2 kali ganda) gene *fece* direkodkan pada pada peringkat awal, 30 minit pertama dan ini mencadangkan bahawa *P. multocida* menggunakan protein periplasmik pada peringkat awal untuk memperolehi besi. Tambahan pula, ekspresi *fece* menurun dari 4 ke -1.5 dengan peningkatan ekspresi gen lain (*fbpb* dan *yfea*) pada masa terkemudian, 60 dan 120 minit mencadangkan bahawa bakteria mengawal keperluan besi mengikut keperolehan besi dengan menurukan ekspresi protein besi. Tambahan lagi peningkatan secara signifikan ($p \leq 0.05$) pada ekspresi *fbpb* (25 kali ganda) pada

minit ke 60 dan ekspresi *yfea* (26 kali ganda) pada masa seawal 30 minit dengan ekspresi tertinggi (42 kali ganda) pada minit ke 120 menunjukan bahawa *P. multocida* strain *PMTB2.1* menggunakan sistem besi berbilang. Hasil kajian ini menunjukkan kepentingan besi dalam daya tahan hidup *P. multocida.*

Kesimpulannya, kajian ini telah memberi pemahaman dalam struktur genom *PMTB2.1* dari segi gen potensi yang berfungsi sebagai faktor virulen dan maklumat patogenomik komparatif yang berharga untuk kajian pada masa akan datang seperti mekanisma yang terlibat dalam keupayaan bakteria tersebut dalam menyebabkan penyakit.

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I certify that a Thesis Examination Committee has met on 13 December 2017 to conduct the final examination of Shagufta Jabeen on her thesis entitled "Complete Genome Sequencing and Analysis of *Pasteurella multocida* Strain *PMTB2.1* and Expression of Selected Genes in Iron-Restricted Environment" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Md Zuki bin Abu Bakar @ Zakaria, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Siti Khairani binti Bejo, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Mohd Zamri bin Saad, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Md. Tanvir Rahman, PhD
Professor
Bangladesh Agriculture University
Bangladesh
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 27 February 2018

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Abdul Rahman Omar, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Zunita Zakaria, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Faez Jesse Firdaus Abdullah, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Nurul Fiza Mat Isa, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
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Universiti Putra Malaysia

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Signature:	
Name of Member of Supervisory Committee:	Associate Professor Dr. Zunita Zakaria
Signature:	
Name of Member of Supervisory Committee:	Associate Professor Dr. Faez Jesse Firdaus Abdullah
Signature:	
Name of Member of Supervisory Committee:	Dr. Nurul Fiza Mat Isa

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ACT	Artemis Comparison Tool
APV	alum-precipitated vaccine
Bp	base pair
BLAST	Basic Local Alignment Search Tool
B2G	BLAST to Gene Ontology
BP	biological process
CC	cellular component
CDs	coding sequences
cDNA	complementary DNA
CGView	Circular Genome Viewer
DNA	deoxyribonucleic acid
^{o}C	degree Celsius
dsDNA	double-stranded DNA
EMBL	European Molecular Bioinformatics Laboratory
GC	guanosine/cytosine
GFF	General Feature Format
GO	gene ontology
HA	hyaluronic acid
HS	hemorrhagic septicemia
Kb	kilobase
kDA	kilodalton
LPS	lipopolysaccharide
min	minute

mL	milliliter
µl	microliter
MF	molecular function
MLST	multi locus sequence typing
Ng	nanogram
nM	nanomolar
NGS	next-generation sequencing
ORF	open reading frame
OM	outer membrane
OAV	oil adjuvant vaccine
PCR	polymerase chain reaction
PM	*Pasteurella multocida*
RNA	ribonucleic acid
RIRDC	Rural Industries Research and Development Corporation
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transcript RNA
Taq	*Thermus aquaticus*
TAE	Tris-acetic-EDTA
VFs	virulence factors
UPM	Universiti Putra Malaysia

CHAPTER 1

GENERAL INTRODUCTION

P.*multocida* is a Gram-negative facultative anaerobic bacterium. It belongs to family *Pasteurellaceae* (Kuhnert and Christensen, 2008) and is an important opportunistic pathogenic bacterium of human and animals. It carries different types of polysaccharides on its capsule and based on that *P. multocida* is grouped into A, B, D, E and F capsular types and further classified into 16 serotypes (1-16) based on lipopolysaccharide antigen (Carter, 1955; Heddleston *et al.,* 1972). *P. multocida* is found as a commensal in many mammals including cattle, buffaloes, domestic cats, dogs and birds in the upper respiratory tract; however, under certain circumstances it can cause opportunistic infections (Boyce *et al.,* 2010). *P. multocida* is associated with a wide range of veterinary diseases in wild and domestic animals of economic significance throughout the world (Harper *et al.,* 2006). In general, *P. multocida* is often associated with chronic as well as acute infections in animals that can lead to significant morbidity manifested as pasteurellosis, pneumonia, atrophic rhinitis, dermonecrosis cellulitis and hemorrhagic septicemia. *P. multocida* can cause pneumonia and respiratory disease in cattle, fowl cholera in avian and hemorrhagic septicemia (HS) and pasteurellosis in cattle and buffaloes.

Pasteurellosis caused by *P. multocida* is an acute septicemic disease characterized by high morbidity and is a high-impact disease in livestock, according to the World Animal Health Organization, Office International des Epizooties, (OIE, 2012). The predominant syndrome of pasteurellosis caused by *P. multocida* in cattle and buffaloes is referred to as bovine respiratory disease (BRD) which is manifested by pneumonic pasteurellosis (Anderson and Rings 2009). In addition, *P. multocida* serogroup A has been implicated in fatal pneumonia of cattle in India (Dabo *et al.,* 2007).

HS is an acute septicemic disease caused by *P. multocida* serogroups B: 2 and E: 2 in cattle and buffaloes and is the most important veterinary bacterial disease of ruminants (De Alwis, 1999; OIE, 2012). Moreover, previous study also indicated the involvement of multiple strains of *P. multocida* in a single outbreak of HS in cattle and buffalo based on molecular study (Bisvas *et al*., 2004) although HS caused by serogroup A strain is only occasionally seen in North America (Rimler and Wilson, 1994; Taylor *et al.,* 1996). *Pasteurella* persist in crypts of the tonsils of experimentally exposed buffaloes and induced carrier animal and is hard to detect as animals remain healthy for several months (De Alwis *et al.,* 1990; Harper *et al.,* 2006). Carrier animals present in the endemic area act as reservoir for new outbreaks. The modes of transmission of the disease were through aerosol or ingestion of feed and water contaminated with infected saliva and discharges.

Over the past 15 year, major advancement has been made in genome sequencing research through the development of next-generation sequencing (NGS) technology

such as 454 Life Science, Solex Illumina, SOLiD and Ion Personal Genome Machine (Quail *et al.,* 2012). This technology has clear advantages over the Sanger sequencing method in term of their high throughput and low cost and the ability to perform multiple parallel analyses simultaneously. Furthermore, with further improvement in NGS technology the development of Pacific Biosciences (PacBio) (English *et al.,* 2012) and Oxford Nanopore Technologies (Timp *et al.,* 2010) based NGS platforms have greatly influenced the field of bacterial genome sequencing . Consequently, not only are the number of completed bacterial genomes increase, but also the coverage and the size of sequenced genomes. Currently, one hundred and twenty one (121) complete or draft *P. multocida* genomes are publicly available in Gene Bank (NCBI, National Center for Biotechnology). However, none of the characterized *P. multocida* genomes were sequenced using Pacific Biosciences except for the recent genome sequencing of *P. multocida* serogroup B: 2 strain Razi 0001(CP017961.1).

The first genomic structure of *P. multocida* was determined by the analysis of the first completely sequenced genome of avian isolate *PM70* (serogroup F) accession no. AE004439.1 (May *et al.,* 2001) which identifies a number of predicted virulence genes and iron uptake genes. At present, only a few genomes have been studied and examined in detail such as *PM36950* (serogroup A) (Michael *et al.,* 2012), PM HB01 (serogroup A) (Peng *et al.,* 2016) and genomes of *P. multocida* strains harboring *P. multocida* toxin (PMT) gene such as PM*HN06* (serogroup D) (Liu *et al.,* 2012).

Currently, the complete genome sequences of *P. multocida* capsular serogroup A, D and F have been generated and characterized and are available publicly as complete or draft genome. However, the complete genome of HS causing serogroups (B: 2 and E: 5) is not available until the recent release of complete genome of Razi 0001 (CP017961.1) a vaccine strain for pasteurellosis in January 2017. Recently, a Malaysian isolate of *P. multocida* serogroup B: 2 strain PMTB have been sequenced as draft genome (AWTD01000000) (Yap *et al.,* 2013). But none of the Malaysian *Pasteurella* strain has been sequenced as a finish high quality complete genome, nor analyzed hence there is no published complete genome sequence of *P. multocida* from Malaysia.Furthermore, *Pasteurella multocida* virulent genes and their interactions in influencing the virulence of the bacteria and pathogenicity in the infected animals are poorly characterized. One of the main reasons is that only a few complete genomes of *Pasteurella multocida* belongs to serogroups A and F has been analyzed and studied in detail and most of the complete genome produced during last decade is present as unpublished data. Also the differences in the genetic structure of the major virulence-related factors between different strains are poorly understood.

Iron is an important nutrient for nearly all life forms. It is possible that iron acquisition in *P. multocida* plays an important role in its survival and pathogenesis in the host, particularly considering that more than 2.5% (53 coding DNA sequences which was later found to be more than 60) of the *PM70* genes are predicted to encode proteins homologous to known proteins involved in iron uptake or acquisition (May *et al.,* 2001; Boyce *et al.,* 2012). Bacterial pathogens, when in a vertebrate host environment, will encounter a depletion of iron, triggering release of the transcriptional control of

ferric uptake regulator (Fur), which represses genes under its control in the presence of iron. Gene expression profiling under iron-limiting conditions based on microarray experiment have identified several iron acquisition genes at increased expression levels, and indeed, different sets of genes appear to be expressed in response to the nature of the iron source (Boyce *et al.,* 2012). In addition, *P. multocida* strains have siderophore-independent iron acquisition systems homologous to the *Actinobacillus* AfeABCD system and the periplasmic binding protein-dependent iron transport systems homologous to *E. coli* FecBCDE and Neisseria FbpABC system (Wilson and Ho, 2013). The presence of multiple iron acquisition systems in *Pasteurella* species may account for their ability to acquire iron under iron limited condition by utilizing certain set of gene under certain conditions.

The general objective of this study is to characterize the complete genome sequence of *P. multocida* strain *PMTB2.1* and to determine the functional importance of selected iron-related genes of the bacteria. Hence, the specific objectives of this study are:

1. To sequence the genome of *Pasteurella multocida* strain *PMTB2.1* based on third-generation sequencing technology using PacBio platform and to perform *de novo* assembly of the generated genome sequences ;
2. To generate and annotate the complete genome sequences of *PMTB2.1* based on a reference genome *P. multocida* strain *PM36950* ;
3. To analyze the complete genome sequences of *PMTB2.1* based on identifications of important sequence motifs and to comparae the genome with other *P. multocida* complete genome sequences by using various bioinformatics tools ;
4. To determine the expression profile of selected iron-acquiring genes of *P. multocida* strain PMTB2.1 grown under iron-restricted environment.

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