

IN SILICO ANALYSIS AND FUNCTIONAL CHARACTERIZATION OF *FHUB*, A COMPONENT OF *ERWINIA MALLOTIVORA* FERRIC HYDROXAMATE UPTAKE SYSTEM

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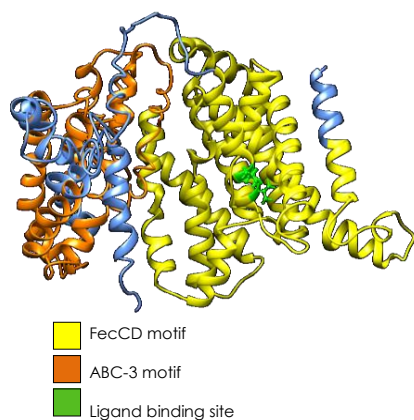
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Graphical abstract



Abstract

Iron is a critical element for bacterial growth as most pathogenic bacteria relies on their host for iron supply. However, iron sources are bounded to the host iron binding protein and specific iron acquisition mechanism is required to chelate and transport the iron to the bacteria. Ferric hydroxamate uptake system or *fhu* is one of the transport systems that import iron in the form of ferric hydroxamate/ferrichrome from the extracellular environment into the bacterial cytosol. In this present study, a detailed *in silico* structural analysis was conducted on an important component of *fhu* transport member from *Erwinia mallotivora* named as *fhuB*. This provide us the structural properties of the protein which includes the domain and 3D model, phylogenetic analysis and the membrane topology. For functional analysis, a knockout mutant of *fhuB* gene strain was generated to evaluate the effect of silencing this gene during *E. mallotivora* infection in papaya. When compared to the wild *E. mallotivora* strain, *fhuB* mutant strain of *E. mallotivora* loss its virulence in causing dieback disease symptom in papaya. The result of this study has revealed the significant role of iron acquisition and metabolism during *E. mallotivora* pathogenesis. This highlights *fhuB* role and importance as the target gene; to inhibit iron uptake in *E. mallotivora* for future study and as a part of future consideration for dieback disease management strategy in papaya.

Keywords: Dieback disease, *Erwinia mallotivora*, *fhuB*, papaya, iron uptake

Abstrak

Ferum merupakan elemen penting bagi pertumbuhan bakteria kerana kebanyakan bakteria patogen bergantung pada perumah untuk mendapatkan bekalan ferum mereka. Walau bagaimanapun, sumber ferum terikat kepada

protein pengikat ferum perumah. Mekanisme pengambilalihan ferum diperlukan untuk mengkelat dan mengangkut ferum ke dalam pathogen. Sistem pengambilan hidroksamat ferum atau dikenali sebagai fhu adalah salah satu sistem pengangkutan yang mengimport ferum dalam bentuk ferik hidroksamat/ferikrom dari persekitaran ekstrasel ke dalam sitosol bakteria. Dalam kajian ini, analisis struktur *in silico* yang terperinci telah dijalankan ke atas komponen penting sistem pengangkutan *fhu* *E. mallotivora* yang dinamakan sebagai *fhuB*. Ini bertujuan untuk mengetahui gambaran struktur protein dan merangkumi domain dan model 3D, analisis filogenetik dan analisis topologi membran. Untuk analisis kefungsiannya, satu mutan *fhuB* *E. mallotivora* dihasilkan untuk menilai kesan gen ini terhadap kepatogenan *E. mallotivora* semasa menyerang pokok betik. Apabila dibandingkan dengan *E. mallotivora* liar, mutan *fhuB* *E. mallotivora* kehilangan kevirulennanya untuk menyebabkan simptom penyakit mati rosot pada pokok betik. Hasil kajian ini menunjukkan peranan penting proses pengambilan dan metabolisme ferum sebagai salah satu mekanisme kepatogenan *E. mallotivora*. Ia juga menunjukkan peranan *fhuB* sebagai salah satu gen sasaran yang boleh digunakan untuk menghalang pengambilan ferum oleh *E. mallotivora*, justeru menentangkannya sebagai salah satu strategi pengurusan penyakit mati rosot bagi betik di masa hadapan.

Kata kunci: Penyakit mati rosot, *Erwinia mallotivora*, *fhuB*, betik, pengambilan ferum

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1.0 INTRODUCTION

Dieback disease in papaya due to *E. mallotivora* infection caused significant loss in papaya production in Malaysia [1]. Similar to other pathogenic bacteria, iron is a crucial nutrients source for *E. mallotivora*. *Erwinia mallotivora* genome sequences has already been obtained via Next generation sequencing. It was reported that 2% of *E. mallotivora* genome were accounted for iron acquisition and metabolism [2], suggesting the significant contribution of this mineral for the growth of *E. mallotivora*. In other reported virulence repertoire of pathogens genomic data, iron acquisition was identified as a key virulence determinant gene for classification of pathogenic bacteria strain [3]. Proteomic study conducted for *E. mallotivora* also highlighted the significant roles of iron transport as part of the bacteria pathogenesis mechanism [4].

Iron is an essential metal for most prokaryotes and eukaryotes due to its utilisation by the organism during redox processes. This enable the organism to participate in many electron transfer reactions [5]. It serves as the key element for various critical metabolic process in organisms such as generation of energy in Tricarboxylic Acid Cycle (TCA)/Krebs cycle, photosynthesis, synthesis of DNA and for defence against reactive oxygen species (ROS). Even so, supply of iron is a problem, especially for aerobic bacteria [6]. Depending on the environment condition, iron can exist in different form. In anaerobic and low pH (<3), iron exist in the soluble form which can be easily taken up by the bacteria without the assistance of chelating agents like

siderophores. However, in aerobic and higher pH (neutral and alkaline condition) environment, irons in Fe^{3+} form are precipitated to form insoluble compounds and thus not readily available [7]. As a part of plant defence against invasion, iron is sequestered by the plant host binding proteins to make it inaccessible for any pathogen. However, successful pathogens were able to overcome and cope with the iron limitation/constraint through their sequestering strategies [8]. Depending on their structure, there are three major types of siderophore; hydroxamates, catecholates and α -hydroxycarboxylates. Each type of siderophores is required and is fitted with specific membrane transport system for them to be transported into the cytosol [9].

One of the important iron binding and uptake system consist of the ferric hydroxamate uptake (Fhu) system which is involved in the uptake of ferric hydroxamate type of siderophores [10]. The Fhu system is composed of four genes namely *fhuA*, *fhuC*, *fhuD* and *fhuB* whereby *FhuA* receptor transports ferrichrome-iron across the outer membrane, whilst periplasmic *FhuD* and cytoplasmic membrane *FhuB* transport ferrichrome and other ferric hydroxamates from the periplasm across the cytoplasmic membrane into the cytoplasm [11].

Structurally, *fhu* membrane transport family are made up of 3 components; a binding protein (BP) located in the periplasm, integral membrane protein (IMP) that is embedded within the cytoplasmic membrane and two nucleotide binding domain (NBD) that are located in the cytoplasm [11, 12]. The IMP which is embedded in the cytoplasm plays an important role in the design and the functionality of

fhu importers. The IMP not only interact with the molecules to be imported into the cytosol, but also with other transport components of the system, where they have binding site for BP (FhuD) and NBD (FhuC) [13, 14]. This factor might contributes to the selection of this component (IMP) as the target for gene knockout or gene silencing for functional study of this transport system family, as well as to effectively hinder the intake of iron into the system. Interestingly, FhuB was shown to be important for virulence activity of several pathogenic bacteria [15].

In our previous study, *FhuB* protein was found to be up-regulated in media cultures of *E. mallotivora* that stimulated the expression of pathogenicity (*hrp*) and virulence proteins of the pathogen *in vitro* [4]. Giving its importance, a research was initiated to further characterize the *fhuB* gene from *E. mallotivora*. This research involved *in silico* characterization and functional analysis of one of the virulence gene of *E. mallotivora*, as an effort to identify target genes to be used in future strategy to combat papaya dieback disease.

2.0 METHODOLOGY

2.1 Retrieval of *EmfhuB* and Related Sequences

The amino amino acid sequence of *EmfhuB* was retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov>) and was downloaded in FASTA format for the *in silico* analysis. The homologous sequences of *EmfhuB* were identified by BLASTp tools in NCBI non-redundant sequence database (<http://www.ncbi.nlm.nih.gov/>).

2.2 Phylogenic Tree Construction

The phylogeny tree was constructed by MEGA Version 7.0 via Neighbor Joining (NJ) method, with p-distance as substitution model via Maximum Parsimony (MP) methods.

2.3 Domain and Protein 3D Model Prediction

Motif finder server (<http://www.genome.jp/tools/motif/>) was used to identify the protein domain. The 3D structure of *EmfhuB* was predicted via SWISS-Model Workspace (<https://swissmodel.expasy.org/>) by selecting the most suited template. The quality of the predicted structure was determined by QMEAN program in the EXPASy server of SWISS-Model Workspace.

2.4 Subcellular Localization and Transmembrane Topology Prediction

Subcellular localization of *EmfhuB* was predicted with TBpred [16] and Gneg-mPLOC [17] server. Meanwhile, the Phobius server (<http://phobius.sbc.su.se/>) was used to predict the presence N-terminal signal

peptides and identification of transmembrane helices of *EmfhuB*.

2.5 Generation of Knockout Mutant ($\Delta EmfhuB$) of *Erwinia mallotivora*

A *fhuB* gene knockout mutant of *E. mallotivora* ($\Delta EmfhuB$) was constructed using the Targetron Knockout system to validate the importance of *fhuB* during the pathogenesis. A combination of gene-specific and intron-specific primers named IBS, EBS1d, and EBS2 that were used to synthesize PCR fragments for the generation of functional cassettes was designed using the Sigma–Aldrich computer-based Targetron algorithm design site (<http://www.sigmagenosys.com/targetron/>). The primers shown below were synthesized by First Base (Malaysia).

Table 1 Primers for $\Delta EmfhuB$ splicing by overlap PCR reaction

IBS1/2	AAAAAAGCTTATAATTATCCTTACACTGCGGGCCAGTGCGCCAGATAGGGTG
EBS1/delta	CAGATTGTACAATGTGGTGATAACAGATAAGTCGGGCCACCTAACCTTACCTTTCTTTGT
EBS2	TGAACGCAAGTTTCTAATTCGGTTCAGTGTGCATAGAGGAAAGTGTCT

The PCR product was treated with *Xho*I (New England Biolabs, Beverly, MA, USA) and *Bsr*GI (New England Biolabs) restriction enzymes and ligated into the pACD4K-C linear vector digested with the same enzymes. For amplification and validation purposes, ligated products were transformed into *E. coli* DH5a strain. Transformants were selected on LB agar plates containing 50 µg/ml kanamycin, and the plasmid DNAs were purified using Qiagen columns. Selection of potential target site and generation of $\Delta EmfhuB$ functional cassettes for targeted gene disruption and splicing by overlap extension PCR were carried out using PCR and sequencing analysis before the generation of the targeted $\Delta EmfhuB$ in *E. mallotivora*.

Erwinia mallotivora competent cells for electroporation were generated based on modified method by Grosser & Richardson (2016). To generate a $\Delta EmfhuB$ strain, the *E. mallotivora* was cultured at 28°C until the culture reached an optical density at 600 nm of approximately 1.0. Bacteria harvested by centrifugation at 12,000 g for 2 min were washed three times with 0.5 M sucrose and then suspended in 0.5 M sucrose. One microgram of the pAR1219 and the plasmid construct for $\Delta EmfhuB$ were electroporated into *E. mallotivora* cells using a Bio-Rad micropulser (Bio-Rad) at 2.5 kV. The transformed bacteria were rescued then cultured on LB agar plates containing kanamycin at 30°C.

2.6 Plant Materials, Bacterial Inoculation and Scoring

The 4-months old seedlings of *Carica papaya* (Ekstotika I) were supplied by the Malaysian

Agricultural Research and Development Institute (MARDI) Pontian, Johor. The seedlings were grown in a greenhouse, where they received 13 h of light a day (30°C). The wild and knockout mutant ($\Delta EmfhuB$) of *E. mallotivora* strains were cultured in LB broth and were left to grow at 28°C incubator shaker until reaching OD₆₀₀ of 1. About 5 mL of *E. mallotivora* culture was injected into the stem of all seedlings at around 15 cm from the shoot area. The scoring was made started from day 3 up to day 30 of post inoculation (dpi).

2.7 Disease Index and Statistical Analysis

Disease severity was evaluated using 5-stage scale; 0 = symptomless, 1 = leaf vein blackening, 2 = leaf vein blackening + slightly wilting, 3 = leaf stalk wilting, 4 = stem blackening and 5 = plant died. The disease severity index (DSI) was computed following the previous study [4]. The experiments were repeated thrice, and the data were analyzed for significant differences via SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA).

3.0 RESULTS AND DISCUSSION

3.1 *EmfhuB* Related Sequences and Phylogenetic Analysis

The ORF of *fhuB* cDNA sequence obtained from the draft genome of *Erwinia mallotivora* BT-MARDI [2] consist of 640 amino acid nucleotides. The BLASTp homology search in the Genbank non-redundant databases had revealed a total of 40 related amino acid sequences (>50% identity), which hit majorly under enterobacteria and g-proteobacteria species. This group of proteins belongs to PRK10577 superfamily that contains the motif of dimer interface, ABC-ATPase subunit interface and putative penicillin-binding protein (PBP) binding regions.

Erwinia mallotivora fhuB (*EmfhuB*) protein sequence showed the highest identity with *fhuB* of *Pantoea coffeiphila* and *Erwinia sp. JUb26* (91%), and 79-78% identity with other bacteria families including *Pantoea rodasii*, *Enterobacter cancerogenus*, *Plautia stali* and *Pantoea rwandensis*. However, low identity percentage showed with three *Erwinia* species including *Erwinia billingiae* (57%), *Erwinia sp. Leaf53* (60%) and *Erwinia sp. B116* (60%) suggested low conservation of *fhuB* at genus level. No other hits from other *Erwinia* species were shown.

Identification of *EmfhuB* homologous sequences (descended from a common ancestor) is important for a reliable evolutionary analysis [18]. A total of 20 *EmfhuB* homologous protein sequence (identity >50%) was used to construct the tree (Figure 1). The branching pattern of the tree showed that *EmfhuB* were well-clustered into three major groups. This branching pattern was supported by high percentage of bootstrap values (>70%) on each node of the major branch. Group 1 and 2 were mainly

made up of enterobacteria species, whereas group 3 was made up of g-proteobacteria species. This suggest the high conservation of *fhuB* protein sequence within the bacteria family. *EmfhuB* was located under group 2 which was highly closely related to *Pantoea coffeiphila* and *Erwinia sp. JUb26*, with bootstrap value of 100%.

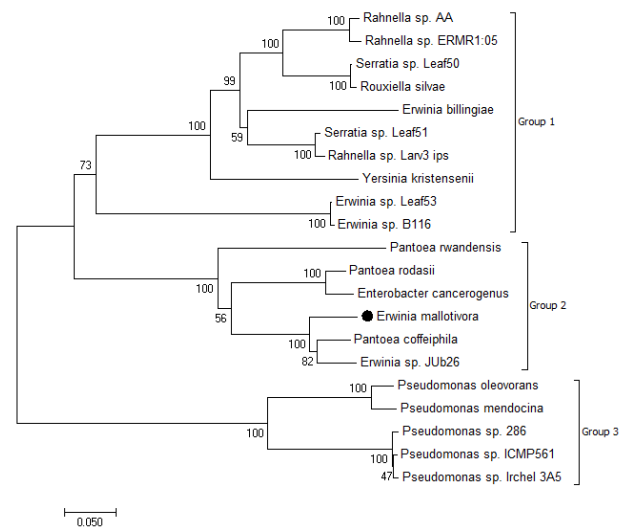


Figure 1 Phylogenetic tree of homologous amino acid sequence of *EmfhuB*

3.2 Domain and 3D Structure

Two transport family motifs, named as FecCD and ABC-3 were detected on *EmfhuB* protein sequence (Figure 2). FecCD motifs were identified in two locations, first which was located near the N-terminal from residue 11 up to 313 and the second at residues 408 up to residue 610, near the C-terminal. The ABC-3 motif spanned from residue 408 to residue 601.

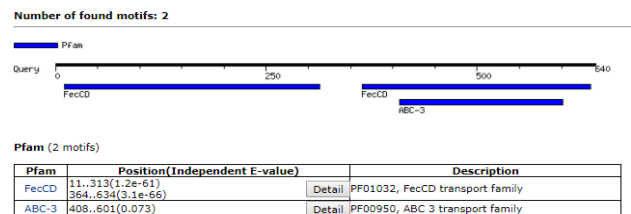


Figure 2 Two functional motifs for the *fhuB* protein of *E. mallotivora*

Homology 3D modelling prediction of *EmfhuB* via swiss-model (Figure 3) disclosed that *EmfhuB* showed the highest identity with 2 chain (Chain A and B) of *E. coli* Vitamin B12 import system permease protein BtuC (PDB ID: [4r9u.1.A](#), X-ray, 2.79 Å resolution). The gene was used as the template for homology modelling. The chain A span from the beginning of N-terminal to residue 314 of *EmfhuB* with 33.70% (GMQE of 0.34, QMEAN of -2.72) identity, whereas the chain

B span from residue 340 to the end of C-terminal with identity of 28.04% (QMQE of 0.35, QMEAN of -3.55). QMEAN [19] estimates the combination of different geometrical properties and provides global (entire structure) and local (per residue) absolute quality of the predicted 3D structure on the basis of single model. Scores of ≥ -4.0 are an indication of models with high quality, which was also highlighted by "thumbs-up" symbol, as shown in Figure 3. This suggests both templates are suitable for *EmfhuB* 3D structure prediction.

Oligo-State		Ligands	GMQE	QMEAN
Monomer (matching prediction)		None	0.35	-3.55
Global Quality Estimate		Local Quality Estimate		Comparison
Template	Seq Identity	Coverage	Description	
4r9u.1.A	28.04%	<div style="width: 28.04%;"></div>	Vitamin B12 import system permease protein BtuC	
Model-Template Alignment				

Oligo-State		Ligands	GMQE	QMEAN
Monomer (matching prediction)		None	0.34	-2.72
Global Quality Estimate		Local Quality Estimate		Comparison
Template	Seq Identity	Coverage	Description	
4r9u.1.A	33.70%	<div style="width: 33.70%;"></div>	Vitamin B12 import system permease protein BtuC	
Model-Template Alignment				

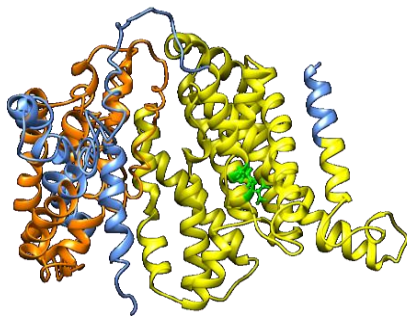


Figure 3 3D model prediction of *EmfhuB*. The ligand binding site is labeled with green color while the yellow color is the FecCD motif. The orange color is the ABC-3 motif

3.3 Signal Peptide, Secretion System and Transmembrane Analysis

The results indicated that *EmfhuB* is localized as an integral membrane protein, specifically at the cell inner membrane (cytoplasmic membrane) of *E. mallotivora*. Via Phobuis, it was revealed that N-terminal signal peptides and transmembrane helices were present in the *EmfhuB* sequence, spanning from nucleotide 1 to 19 (Figure 4). A signal peptide which is a short leader sequence (normally 16-30 amino acids long) are located at the N-terminus of the newly synthesized protein.

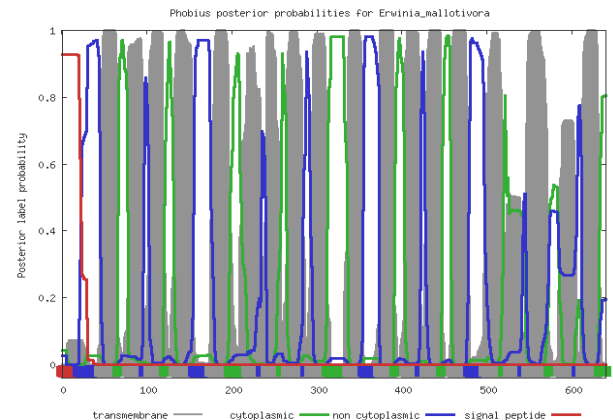


Figure 4 Prediction of the presence of transmembrane domains and signal peptides via Phobius server. The x-axis shows the number of the amino acids of the specific *EmfhuB* sequences

3.4 Transmembrane Topology of *EmfhuB*

The prediction of membrane topology provides the most probable location on where the soluble domains are oriented relative to the plane of the membrane. This offers guidance for further experiments to identify functional domains of membrane proteins.

Cytoplasmic transmembrane arrangement showed that *EmfhuB* contains 16 α -helix transmembrane region which is also known as hydrophobic region (8 at each halves of N- and C-termini) which are connected by 7 positively charged loops facing the cytosol (cytoplasmic region) and 8 loops facing the periplasm (non-cytoplasmic region). Both the N- and C-terminal end were facing the cytosol (Figure 5). The loop regions that are exposed to the periplasm and cytosol are suggested as the putative interaction site of *fhuB* with the periplasmic binding protein (FhuD) and ATP-hydrolase binding protein (FhuC), respectively. Compared to the well-studied/ model *fhuB* of *E.coli*, *EmfhuB* consist of 20 transmembrane regions (10 at each halves).

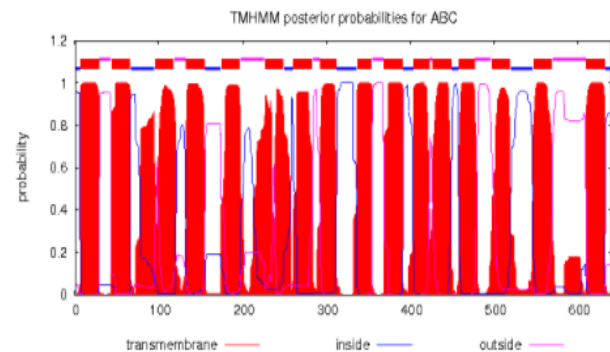


Figure 5 Predicted topology of *EmfhuB* protein

3.5 Construction and Molecular Validation of $\Delta EmfhuB$ Strain

Construction and molecular validation of $\Delta EmfhuB$ were carried out using Targetron Gene Knockout System. Identification of binding Ltr group II intron site within the *EmfhuB* gene sequence for the generation of mutated PCR products were carried out using the algorithm located at Sigma Aldrich targetron website (www.sigmaldrich.com/targetron). Primers identified from the analysis were synthesized and used to generate PCR fragment in a splicing by overlap PCR reaction. Generation of *EmfhuB* functional cassettes for targeted gene disruption and splicing by overlap extension PCR were carried out as shown in Figure 6. The PCR utilizing the primers in Table 1 to mutate intron at several positions generated a PCR fragment of 350 bp in size with *Hind*III and *Bsr*GI restriction enzymes at the 5' and 3' UTR region. Plasmids containing the targeted fragments were amplified and digested with *Hind*III and *Bsr*GI restriction enzymes and sub cloned into the pACD4K-C linear vector to generate construct for the targeted $\Delta EmfhuB$.

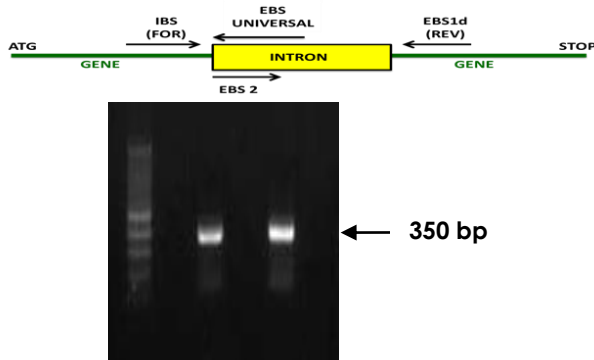


Figure 6 Schematic presentations and PCR analysis carried out for the generation of *EmfhuB* splicing and overlapped PCR products for the generation of $\Delta EmfhuB$ mutants

TargetTron system plasmids require the use of T7 promoter for targeting and mutation of selected genes into the genome of the bacteria of interest. Since *E. mallotivora* does not express T7 RNA polymerase, pAR1219, a pBR322-based vector that expresses T7 RNA Polymerase under the control of the IPTG inducible lac UV5 promoter was used during the TargetTron Gene Knockout activity. Transformation of the plasmid pAR1219 was carried out to obtain the T7 promoter that can be used to express the intron and to disrupt the chromosomal genes in *E. mallotivora*. *Erwinia mallotivora*-pAR1219 strains were successfully transformed with the effectors functional cassette for the gene via electroporation and chemically competent cells transformations. Positive transformants with mutations which were introduced in *EmfhuB* were detectable after 48 hours selection on kanamycin plates. Intron insertions were detected by PCRs with genomic DNA isolated from the

transformed colonies by using combinations of gene specific and intron-specific primers for verification. The positive transformants were later verified by DNA sequencing. Confirmation of the $\Delta EmfhuB$ mutants were also conducted via PCR colony performed using specific primers targeting *E. mallotivora* HrpN, Isochorismate mutase and HrpS genes of as shown in Figure 7. This is to further confirm that the targeted knockout was carried out in *E. mallotivora* strain.



Figure 7 Analysis of *E. mallotivora* mutants using *E. mallotivora* mutants specific primers to confirm the targeted mutation were carried out in *E. mallotivora* strains. Three specific primers that corresponded to HrpN, Isochorismate mutase and HrpS *E. mallotivora* were used to confirm that the $\Delta EmfhuB$ mutants were targeted in *E. mallotivora* strains

3.6 Involvement of *fhuB* in the Pathogenicity *E. mallotivora*

EmfhuB deletion mutant of *E. mallotivora* ($\Delta EmfhuB$) was successfully generated via TargetTron Gene Knockout System. To evaluate the contribution of *fhuB* in *E. mallotivora* pathogenicity, wild-type and $\Delta EmfhuB$ *E. mallotivora* strain was inoculated to papaya seedlings. The scoring on the progression of dieback disease severity index on papaya seedlings (Table 2) showed that the stage 1 disease symptom (blackening of leaf vein) was detected as early as 3 days post infection (dpi) in plants inoculated with wild-type *E. mallotivora*. The progression of disease continued until 20 dpi, where at this stage the plants completely died. In contrast, no disease symptoms were detected in plants inoculated with $\Delta EmfhuB$ (scoring was made up to 30 dpi). This result suggested the importance of *fhuB* for *E. mallotivora* pathogenicity. Knockout mutation of this gene might lead to iron deprivation that eliminates the virulence of *E. mallotivora*.

However, besides *fhu* transport system, the presence of other iron transport systems including Btu (Vitamin B12 uptake system), Feo and efe (Ferrous iron transport system) were also identified in the *E. mallotivora* genomic data [2]. This has raised a question on how a knockout of a gene in one of the iron transport system could lead to the complete loss in the virulence of *E. mallotivora*. At this point, our result suggest that the ferric-hydroxamate system might be the sole or major source of iron for *E. mallotivora*. To confirm this, further experiment on the growth of mutated *EmfhuB* strain in various sources of

iron-restricted media could be conducted. This is in accordance to a report by Speziali *et al.* 2006 for the bacteria *S. aureus* [20]. The deletion of *fhuB* had resulted in the inability to transport or utilize any form of ferric-hydroxamate complexes including ferriochrome, Desferal, coprogen and aerobactin [20]. In another report, mutation of *fhu* operon for *Yersinia pestis* resulted in deficient uptake of iron [9].

Table 2 The dieback disease severity scoring in papaya seedlings inoculated with wild and *fhuB* knockout mutant *E. mallotivora*. (Stage 0 = symptomless, Stage 1 = leaf vein blackening, Stage 2 = leaf vein blackening + slightly wilting, Stage 3 = leaf stalk wilting, Stage 4 = stem blackening and Stage 5 = plant died)

Strain of <i>Erwinia mallotivora</i>	Scoring of infection						
	Day-3	Day-6	Day-9	Day-12	Day-16	Day-20	Day-30
Wild type	1	2	2	3	4	5	5
Knockout mutant ($\Delta EmfhuB$)	0	0	0	0	0	0	0

4.0 CONCLUSION

In this study, we have identified *in silico* structural properties of *fhuB* proteins as well as its critical role in affecting the virulence of *E. mallotivora*. In these studies, *fhuB* genes were characterized using bioinformatic tools and was shown to contain all elements of *fhuB* motif. $\Delta EmfhuB$ was successfully generated via Targetron system and the effect of knockout strain was observed through plant screening method using plant infection assay. The outcomes of this project will have important implications in defining the pathogenesis of *E. mallotivora*.

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