CONSTRUCTION OF KNOCK-OUT *FUR* GENE FOR THE PRODUCTION OF IROMPs IN *PASTERURELLA MULTOCIDA* 6: B FROM CATTLE

BY

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Abstract

Haemorrhagic Septicaemia (HS) is a major disease of cattle and buffaloes characterized by an acute, highly fatal septicaemia with high morbidity and mortality. In Asia, HS caused by infection with *Pasteurella multocida* serotype B:2 or 6:B. To overcome the problem associated with the use of currently available vaccine the quality and effectiveness of vaccine need to be improved. The production of IROMPs is regulated by Fur protein by repressing the expression of the respective genes when complexed with iron. Fur (ferric uptake regulator) protein is a DNA-binding protein which regulates iron-responsive gene. The inactivation or deletion of *Fur* gene avoids the repression effect of the transcription of the downstream sequence of the genome which eventually leads to uncontrollable production of IROMPs. Therefore, it is predicted that all IROMPs will be produced in *fur*-knockout strain when grown in BHI and has a good potential to be used to prepare a subunit haemorrhagic septicamia vaccine based on IROMPs.

Introductions

Haemorrhagic Septicaemia (HS) is a contagious disease caused by *Pasteurella moltocida* type 6: B mainly of cattle and buffalo in South East Asia including Malaysia (Heddleston et al., 1972). *P. multocida* is a small, Gram-negative bacterium. It is a non-motile coccobacillus and is penicillin-sensitive (Confer et al., 1996). The pathogenic parts are polysaccharide capsule, lipopolysaccharides (LPS), outer membrane proteins (OMPs), fimbria, adhesins, endotoxines and extracellular enzymes (Confer, 1993). *P. multocida* is transmitted by direct contact with infected animals and on fomites. Cattle and buffalo become infected when they ingest or inhale the causative organism, which probably originates in the nasopharynx of infected animals. The global distribution of the disease and the wide variety of livestock affected account for considerable economic losses due to this pathogen worldwide (Confer, 1993).

Fur gene

Fur (ferric uptake regulator) protein is a DNA-binding protein which regulates iron-responsive genes (Hernandez et al., 2002). The inactivation or deletion of *Fur* gene avoids the repression effect of the transcription of the downstream sequence of the

genome which eventually leads to uncontrollable production of IROMPs. Therefore, it is predicted that all IROMPs will be produced in *fur*-knockout strain when grown in BHI and has a good potential to be used to prepare a subunit HS vaccine based on IROMPs. The knowledge of the iron-uptake is an important aspect of the biology of this organism because it is known that cultures grown under conditions of iron depletion can induce cross-protection against any challenge with *P.multocida* virulent strains (Ruffolo et al., 1994).

Materials and Methods

Bacterial, Plasmid and Reagent

Pasteurella multocida bacteria used in this study is a local isolate stored in 20% glycerol stock at -80°C. Plasmid used in this study is pMUTIN4. Plasmid DNA was purified using Gel Extraction Kit and Purification Kit (QIAGEN) following the manufacturer's instruction and stored in TE buffer at -20°C.

Primer design

Primer was design for two fragments of *Pasteurella multocida* which A (840 bp) and B (1088bp). Forward primer (PMfur A01)**5´-AATGCGAATTCGTGGTGGCAGATATCCCGGG-3´**.Reverse primer (PMfurA02) **5´-TATGACCATGCAACTGAACACCACGATCAC-3´**. Forward primer (PMfurB01) **5´-TATGACCATGGGTGATCGTGGTGTTCAGTTG-3´**. Reverse primer (PMfurB02) **5´-ATTCTGGATCCATACTCGCAAAGTTGGACGC-3´**. Primer design for Kanamycin was Forward primer (FKan) **5'-GGCGGCCATGGCAGCGAACCATTTGAGG-3'**. Reverse primer (RKan) **5'-GGGGCCCATGGAATTCCTCGTAGGCGCTCGG-3'**. The primer design and bioinformatics studies were fulfilled by aid of three softwares such as Redasoft Visual Cloning 2000, OligoExplorer1.2 and OligoAnalyzer1.2 and the blast will utilize software available in the World Wide Web.

DNA techniques

Amplification of Fragment A and Fragment B was performed using *Taq* DNA polymerase (Invitrogen) for 40 cycles consisting of 2 min at 95°C (DNA denaturation), 45 sec at 60°C (primer annealing) and 1 min at 72°C (DNA synthesis). PCR of Kanamycin also using *Taq* DNA polymerase from Invitrogen for 39 cycles consisting of 2 min at 94°C for DNA denaturation, 1 min at 62°C for primer annealing, and 3 min at 72°C for DNA synthesis.

Construction of Fur-mutant strain of Pasteurella Multocida type 6:B

In order to develop HS vaccine that contains IROMPs, *fur* gene that responsible in expression of Fur protein in *Pasteurella multocida* will be deleted using an insertional replacement of the gene with antibiotic resistant marker such as kanamycin cassette. This

is by cloning of the gene marker into the middle of the *fur* gene during the construction of mutagenesis plasmid. The construction of the plasmid for mutagenesis will involve the step such as amplification of *fur* by PCR from genomic DNA *P. multocida* type 6:B, creation of an internal restriction site and cloning into the suicide shuttle vectors (pMUTIN4 or pAZ106) in *E.coli* cloning system. As shown in Figure 1, two sets of primer were designed in order to separate *fur* sequence into two parts. The two fragments namely Fragment A and Fragment B.

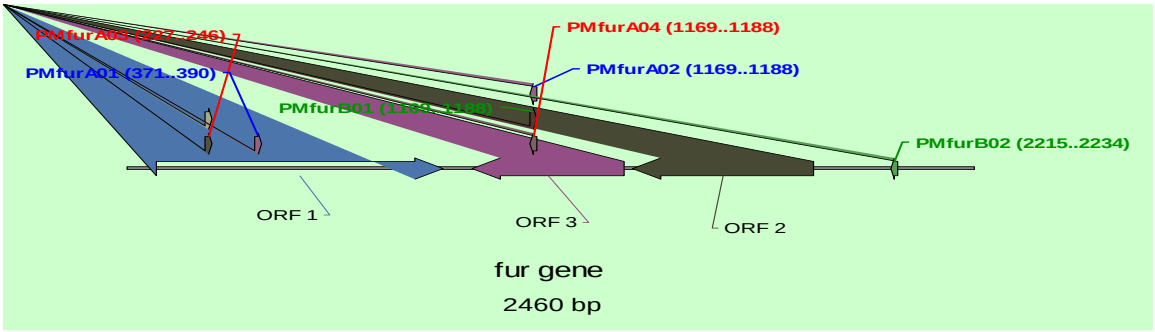

Figure 1

pMUTIN4 (Figure 2) is the plasmid vector that was utilized for the development of the recombinant plasmid. It replicates in *E.coli* via the *Col*E1 origin with selection via ampicilin resistance. Having a multiple cloning sites (MCS) and bears a modified *lacZ* reporter gene downstream from the MCS enable transcriptional fusion with the cloned inserts.

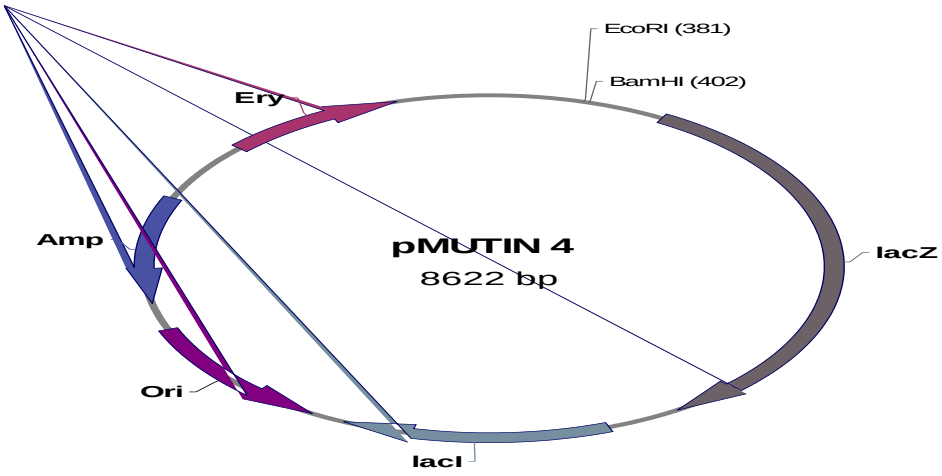

Figure 2

pMUTIN4 was cut with restriction enzymes *Eco*RI at position 381 bp and *Bam*HI at position 402 bp. The selection of restriction enzymes *Eco*RI and *Bam*HI is due to their

single recognition site in the whole pMUTIN4 sequence. This enables sites for introducing the insert sequence into the vector sequence. The inserts consist of three fragments; Fragment A (1.5kb), Fragment B (1.0kb) and kanamycin sequence (1.5 kb). Thus, the expected size of the recombinant plasmid to be developed is approximately 12.1 kb as illustrated in Figure 3.

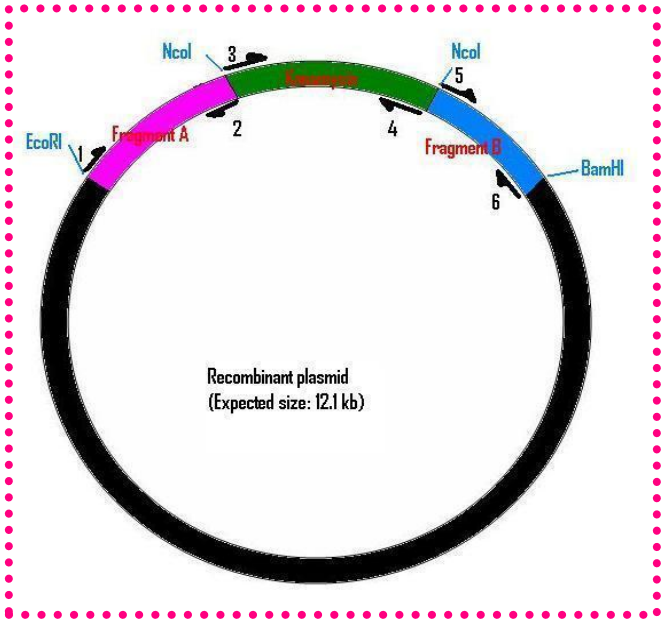

Figure 3

Results and Discussions

PCR was carried out using primer PMfur A and PMfur B. Another set of primers was taken from other research project to amplify a sequence of antibiotic resistance (kanamycin). The kanamycin resistance gene was inserted in between the fragments, thus causing the inactivation or deletion of the *fur* gene. Kanamycin resistance in alternative will act as an indicator for the successful recombinant colonies. As shown in Figure 4, Fragment A, Kanamycin and Fragment B will be ligated at sticky ends of restriction enzyme *Nco*I. The fur gene will first be modified by introducing restriction sites for *Eco*RI, *Nco*I and *Bam*HI into the gene sequence to fix with the restriction sites available in the suicide vector, pMUTIN4.

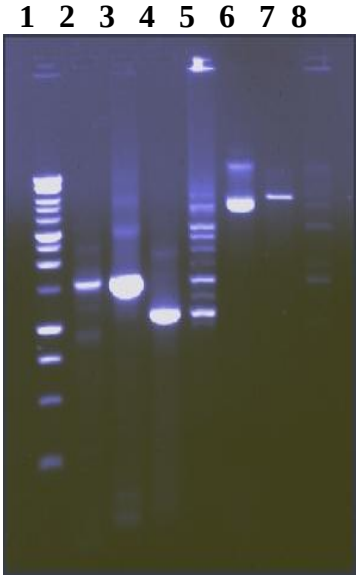

Figure 4

Construction of Fur-mutant strain
Lane 1 DNA ladder with its appropriate sizes
Lane 2 Fragment A
Lane 3 Kanamycin
Lane 4 Fragment B
Lane 5 Ligated AKB
Lane 6 Digested pet24d
Lane 7 Ligated AKB and pet24d.

Because of the problem occur with the pMUTIN4, the gene was cloned into plasmid pet24d (5.2 kb) in *E.coli* Top 10 first. Expected size of the recombinant plasmid was 9.0 kb, shown in Figure 5. A single colony of *E.coli* Top 10 with the recombinant plasmid (Clone AZ001) on LB agar was inoculated into LB broth. Confirmation of the Clone AZ001 was carried out by extracting the plasmid from the successfully survived single colony of *E.coli* Top 10. Then, the plasmid was digested with restriction enzyme followed by agarose gel electrophoresis. In Figure 6, only digestion with EcoRI gave the one band of 9.0 kb. Whereas did not satisfy with the expected size of restriction digest analysis.

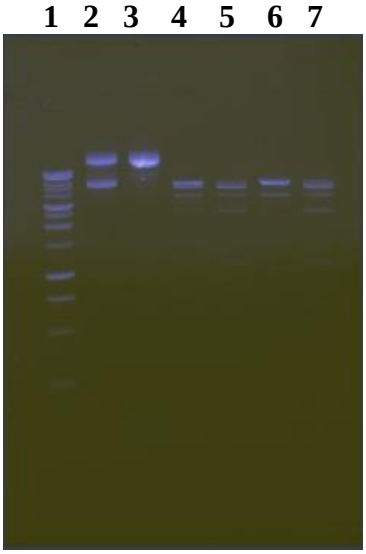

Figure 5

Development of recombinant plasmid.
Lane 2 and 3 Clone AZ001
Lane 3, 4, 5 and 6 Restriction digest Clone AZ001 with EcoRI and BamHI.

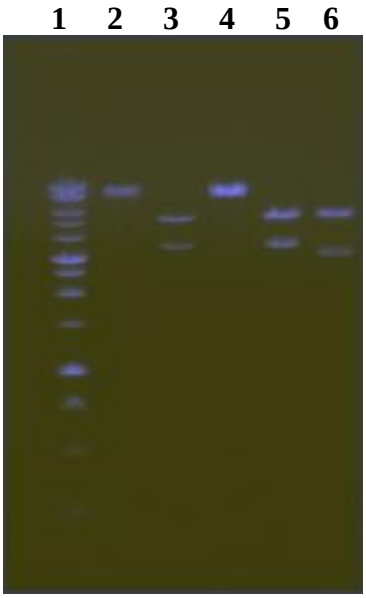

Figure 6

Restrictions digest analysis for conformation of Clone AZ001.
Lane 2 EcoRI
Lane 3 BamHI
Lane 4 NcoI
Lane 5 BamHI and EcoRI
Lane 6 BamHI, EcoRI and NcoI

Conclusions

The Clone AZ001 still under analysis in order to confirm it as a successful recombinant plasmid. The study will be continued by transforming the construct into pMUTIN4 competent cells by electrophoration and the transformants could be selected using antibiotic and verified by PCR.

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