INACTIVATION/DELETION OF *PLD* GENE FROM *Corynebacterium pseudotuberculosis*

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Abstract

Caseous lymphadenitis is a disease caused by *C. pseudotuberculosis* which brings significant economic losses to small ruminant producers worldwide. The *pld* gene that encodes potent exotoxin has been considered as the major virulence factor for this bacterium. Deletion of *pld* gene is done by inserting a foreign sequence (*Kanamycin* gene) in between the *pld* sequence, thus eliminating the original sequence of the *pld* gene. In the future, the mutant strain produced from this study can be used for vaccine production.

Key words: *Corynebacterium pseudotuberculosis*, caseous lymphadenitis, *pld* gene

Introduction

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis (CLA or cheesy gland) in goat and sheep (Batey 1986). This gram-positive facultative intracellular bacterium is found on fomites and in soil and manure that is contaminated with purulent exudates from animals. Infection occurs after *C. pseudotuberculosis* penetrates through unbroken or abraded skin or through mucous membrane, following which the bacteria are carried to the local lymph node. Once established, they multiply to cause inflammation, necrosis and abscessation of the superficial lymph nodes. The infection can cause important economic losses for ovine and caprine husbandries, due to reduced wool, meat and milk yields, culling of affected animals and condemnation of carcasses and skins (McNamara et al., 1995; Hodgson et al., 1990).

Phospholipase D activity is not only found in bacteria (Hodgson et al. 1990), it has been identified in a wide variety of cell types and organisms such as plant (Wang et al. 1994) and mammals (Exton 1990). The *pld* gene encodes an exotoxin that probably promotes bacterial dissemination, increasing vascular permeability (Hodgson et al., 1994) through the hydrolysis of ester bonds in sphingomyelin in mammalian cell membranes following infection (Dorella 2006). In this study, *pld* gene from local *C. pseudotuberculosis* isolate will be inactivated by allelic replacement with antibiotic marker gene (*Kanamycin*). After PCR amplification and ligation between two *pld* gene fragment and *Kanamycin* gene, the gene will be cloned into suicide plasmid pMUTIN4 in *E. coli* Top 10.

Materials and Methods

Bacteria, plasmid and Reagent

C. pseudotuberculosis bacteria used in this study is a local isolate stored in 20% glycerol stock at -80°C. The genomic DNA was extracted from this bacteria using DNeasy® Blood & Tissue Kit. Plasmid that is used in this study is pMUTIN4. Plasmid DNA will purify using QIAquick® Gel Extraction Kit (Qiagen) following the manufacturer's instruction and stored in TE buffer at -20°C.

Primer design

Two sets of primer were needed in order to separate *pld* gene sequence into two parts, namely as Fragment A an Fragment B. In this study to separate this gene into two parts, two sets of primers, namely pldf_a_2 and pldr_a_2 for Fragment A, pldf_b_2 and pldr_b_2 for Fragment B were designed.

Another set of primers was taken from previous studies to amplify a sequence of antibiotic resistance gene (*Kanamycin*). The *Kanamycin* resistance gene was inserted in between the fragments, thus causing the inactivation or deletion of the gene. Kanamycin resistance alternatively also acts as an indicator for the successful recombinant colonies. The primer design and bioinformatics studies were conducted using 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 35 cycles consisting of 5 min at 95°C (DNA denaturation), 1 min at 60°C (primer annealing) and 1 min at 72°C (DNA synthesis). PCR of *Kanamycin* gene 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. The PCR products were purified using QIAquick® Gel Extraction Kit (Qiagen) accordance with the manufacturer's instruction.

Nucleotide sequencing and computer analysis

For conformation, Fragment A, Fragment B and Kanamycin gene that were obtained from PCR reaction have been sent to 1st Base for sequencing. Every fragment that is successfully sequenced will be blast using NCBI.

Result and Discussion

The deletion of the *pld* gene can be done by removing the whole sequence of the gene or by inserting a foreign sequence in between the *pld* sequence, thus eliminating the original sequence of the *pld* gene. The recombinant fragment will be cloned into pMUTIN4 suicide plasmid in Top 10 competent cells. Then, the recombinant plasmid will be extracted, purified and transformed into *C. pseudotuberculosis* competent cell.

The *pld* gene encoding PLD protein with the size of 1638bp was obtained from GenBank accession number: L16587. Restriction enzymes used in this study is unique to the site of pMUTIN4 and there are no restriction enzymes at cutting sites within the *pld* gene for BamH1, Nco1 and HindIII. Both gene knockout and Kanamycin primer were then designed by introducing those restriction enzymes at the site.

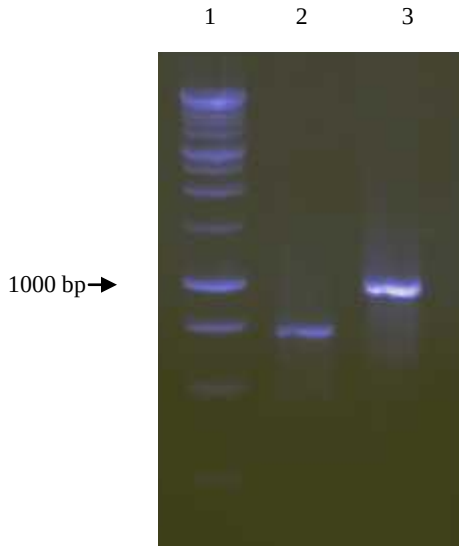

Figure 2: PCR product of *pld* gene from *C. pseudotuberculosis*

Lane 1: 1kbp DNA ladder (Promega)
Lane 2: Fragment A
Lane 3: Fragment B

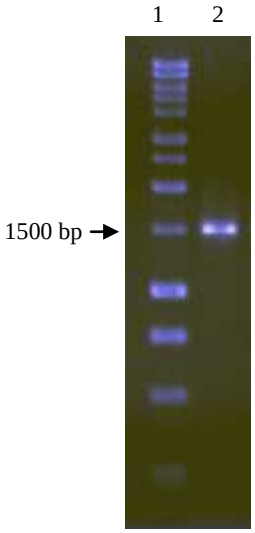

Figure 3: PCR product of Kanamycin gene

Lane 1: 1kbp DNA ladder (Promega)
Lane 2: Kanamycin gene

A PCR reaction of Fragment A and Fragment B was performed using genomic DNA from *C. pseudotuberculosis* as a template, with the expected size of 638bp and 1000bp respectively. Both fragments were successfully amplified (Figure 2). Amplification of Kanamycin gene was performed using *S. aureus* SHRM04 *isdB: Tet fur: Kan* from Ramlan previous study. Kanamycin gene with expected size of 1.5kb also successfully amplified (Figure 3). The DNA fragment was purified using QIAquick®Gel Extraction Kit. Both purified fragment then digested with HindIII and Nco1 for Fragment A and Nco1 and BamH1 for Fragment B. Kanamycin gene also digested using Nco1 restriction enzymes.

pMUTIN4 is the plasmid vector (Figure 4) 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 (Vagner et al., 1998).

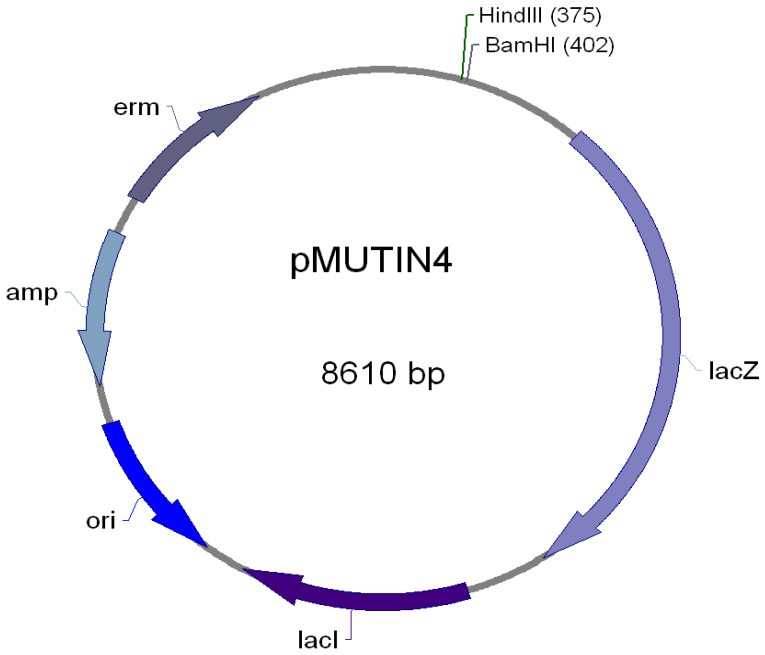

Figure 4

pMUTIN4 will be cut with restriction enzymes HindIII at position 375 bp and BamH1 at position 402bp. The vector becomes a linearized DNA with HindIII and BamH1 sticky ends with the length of 8601bp. The selection of restriction enzymes HindIII and BamH1 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 (638bp), Fragment B (1000bp) and Kanamycin sequence (1.5 kb). Thus, the expected size of the recombinant plasmid to be developed is approximately 11.7kb**.** Fragment A, Kanamycin sequence and Fragment B will be ligated at sticky ends of restriction enzyme NcoI. Restriction analysis on the pMUTIN4 sequence generates zero of NcoI restriction cutting site. This principle that leads to the use of this enzyme for ligating the fragments together.

Conclusion

Currently this work is still in progress to obtain the desired recombinants between Fragments A, B and Kanamycin gene where the estimated size is 3138bp.

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