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Chemical Mutagenesis, Isolation and Characterization of Non-Haemolytic

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Abstract

A collection of non-haemolytic *M. haemolytica* mutants were generated by EMS (ethyl methanesulfonate) mutagenesis. Total proteins from these mutants were examined by Western immunoblot analysis using antibodies specific for the leukotoxin *LktA*. *LktA* was not detected in some of the mutants as expected; however, *LktA* was detected in several mutants in spite of a non-haemolytic phenotype. These mutants could have a defect in any one of the four genes, or elsewhere in the genome suggesting other factors may be required for fully functional leukotoxin production. The *lktA* gene from mutant 36 was isolated by PCR and sequenced. Mutant 36 was found to have a G-C to A-T substitution resulting in codon change from GAG to AAG and E470K amino acid substitution.

INTRODUCTION

Mannhemia haemolytica A1 (*Mh*) is a bovine pathogen that causes pneumonic pasteurellosis resulting in significant economic loss for the cattle industry [1,2]. The most important virulence factor produced by the bacterium is the leukotoxin (Lkt) which is a pore-forming cytolysin specific for bovine leukocytes but non-specific for erythrocytes resulting in cell lysis [3-6]. The non-specific binding and lysis of erythrocytes by Lkt produces a zone of clearance on blood agar plates. Lkt is produced by a four gene operon (*lktCABD*) which has been cloned and sequenced [7-9]. *lktA* codes for the structural protein *LktA*, *lkt*C codes for an activator enzyme that is responsible for acylating *LktA* to its active form [7,8]. Together, *lkt*B and *lkt*D code for a secretion apparatus that secretes the toxin extracellularly [9].

The *lkt* operon showed extensive homology with the α-haemolysin (*hly*) operon in *Escherichia coli* [10]. Both toxins were found to have amino acid repeats within the toxin involved in binding of Ca^{+}[11]. The term RTX (repeats in the toxin) was coined to represent this family of toxins [12,13]. Subsequently, RTX toxins have been discovered in many bacterial pathogens such as: *Actinobacillus pleuropneumoniae, Actinobacillus suis, Aggregatibacter actinomycetemcomitans, Bordetella pertussiss, Nesseria meningitidis* [14-18]. Since then a lot of information has been gathered on the *Mh* leukotoxin and related RTX toxins, including binding specificity to target cells, pore forming activity, immunogenicity and the use of the toxin as vaccine components [13,19-22]. However, very little is known about the genetic mechanism(s) that regulate expression of the *lkt* operon. We have previously conducted studies which showed that temperature, pH, Fe^{2+} regulates *lkt* expression in *Mh* [23]. Studies by Highlander suggested bending of DNA at the *lkt* promoter, possibly involving IHF and a transcriptional factor [24-26]. Another report by Kolodrubetz on the human periodontal pathogen *A. actinomycetemcomitans* also suggested the interaction of a positively acting factor at its *lkt* promoter [27,28]. Therefore, it is very likely that some positively acting transcriptional factor is involved in expression of the *lkt* operon.

In this study, we attempted to search for genetic factor(s) involved in the regulation of expression of the *Mhlkt* operon. We utilized the phenotypic property of *Mh* which produces a zone of clearance on sheep's blood agar plates due to haemolysis of red blood cells by the leukotoxin. Hence any mutation which abolishes Lkt activity or production will result in a non-haemolytic phenotype. The chemical mutagen ethyl methanesulfonate (EMS) was chosen to mutagenize *Mh* as this mutagen has been shown to induce primarily base-substitutions [29]. Here, we report the successful mutagenesis and isolation of non-haemolytic *Mh* mutants and the sequence analysis of mutant 36. We showed that mutant 36 harbours a G-C to A-T substitution resulting in an amino acid substitution in a highly conserved position of the toxin.

MATERIAL AND METHODS

Bacterial strains, culture conditions and EMS mutagenesis

Mh A1 is from our laboratory collection and is maintained on 5% sheep's blood agar plates. For liquid cultures, bacterium is grown in BHI broth with shaking at 37C. The EMS mutagenesis

procedure is modified from the protocol for *E. coli* according to Miller [30]. First, a kill/survival curve is established. Briefly, an overnight culture of *Mh* is sub-cultured 1/200 in 20 mL BHI broth and grown for 4 hr. The cells were collected by centrifugation, washed once in the same volume of 0.1 M $MgSO_4$ and re-suspended in half the volume (10 mL) of 0.2 M Tri-HCl (pH 7.5). Two mL aliquots were transferred into 15 mL sterile screw-cap plastic tubes and various amounts of EMS (Sigma Chemicals M0880-5G) were added. The tubes were placed horizontally and cells were mutagenized for 2 hr at 37 C with shaking. Afterwards, 0.1 mL was recovered for serial dilution and plated for survivors. One mL of the remaining culture was diluted into 9 mL BHIB and grown overnight. The recovered cultures were serially diluted, plated on sheep's blood agar plates for non-haemolytic phenotype. Non-haemolytic colonies were picked and streaked for single colonies to confirm the mutant phenotype.

SDS-PAGE and Western immunoblot analysis

Total proteins from *Mh* cultures were examined by SDS-PAGE and Western immunoblot using our standard laboratory procedure. An equal volume of overnight culture in BHI broth was mixed with 2X sample buffer, boiled and separated by SDS-PAGE electrophoresis. Afterwards, the proteins were transferred to nitrocellulose membrane by electro-blotting. The proteins were immunostained with either monoclonal antibody 601 [31] or a polyclonal antibody [21] against *LktA*. The bound antibodies were then visualized with the appropriate second antibody (goat anti-mouse or goat anti-rabbit, respectively) conjugated to alkaline phosphatase and colour development accordingly. Some of the gels were also stained with Coomassie Blue after electrophoresis to provide visualization of proteins on the gel.

PCR amplification and sequence analysis

Total bacterial DNA was isolated using the QIAGEN Genomic-tip midi-prep according to the protocol. PCR was carried out using our standard laboratory conditions in an Eppendorf Mastercycler machine. Briefly, PCR were carried out in 25 µL reactions containing (final concentration) 1X PCR buffer, 2mM $MgCl_2$, 0.4 mM of each dNTP, 100 µM of both forward and reverse primers, 2.5 units of Platinum *Taq* DNA polymerase (Invitrogen) and 0.5 µL template DNA. A typical PCR cycle included: a onetime hot-start at 94 C for 2 minutes; 30 cycles of denaturation at 94 C for 1 minute, annealing at 50 C for 1 minute, extension at 72 C for3 minutes and a final extension at 72 C for 5 minutes. The primers (Table 1) were designed based on the published sequence of the *lkt* operon to amplify overlapping fragments to ensure the entire *lktA* region is sequenced. Three fragments of approximately 780 bp, 1.9 kbp and 1 kbp were amplified for sequence analysis. The PCR products were purified using the QIA quick PCR Purification Kit (QIAGEN), eluted in 30 µL double distilled H_2O and sent for sequence at the Genomic Facility, Advance Analysis Centre, University of Guelph, using the dye terminator sequencing method. The sequence data were examined with version 1.4 of the Finch TV software program. Finch TV was used to convert the sequence data into a FASTA file and compare to the published *lkt* sequence (Gene Bank access #M20730) using the Clustal W2 software program which highlights discrepancies in the aligned sequences.

RESULTS AND DISCUSSION

EMS mutagenesis

The base-substitution mutagen was used for this study to isolate mutant(s) that affect leukotoxin production and activity. This mutagen was selected in favor of other mutagens to avoid any gross alterations such as deletions or rearrangements in the DNA. A kill curve was established to determine the proper dosage for mutagenesis on *Mh*. It has been suggested that a 99.9% killing is the desired dose for EMS mutagenesis in *E. coli* [30]. The results in Figure (1) showed a kill curve based on three separate experiments and that a dose of 5 µL EMS was used for subsequent mutagenesis experiments. This protocol for EMS mutagenesis is simple and straight forward and can be adapted for mutagenesis with a suitable selection scheme.

Isolation of non-haemolytic mutants

The zone of clearance by *Mh* on blood agar plates gives it a simple phenotype to visualize non-haemolytic mutants. The results in Figure (2) showed the contracting non-haemolytic colony phenotype in comparison with the haemolytic parent. From four separate mutagenic experiments, thirty two mutants were recovered. Depending on when the mutagenic event took place during the overnight culture, it is possible that some of the mutants from each experiment are clones of each other. Mutants from separate experiments should be result of different mutagenic events.

Western Immnuoblot analysis of LktA

To determine whether the mutants are capable of producing the leukotoxin, total proteins from the mutants were examine by SDS-PAGE and Western immunoblot using antibodies against *LktA*. The results in Figure (3) showed that there is no *LktA* produced in mutants 1 and 17 as expected, but mutants11, 12, 14, 15, 34, 36 still produced *LktA*. Mutants 12, 14 and 34 appear to have a reduced amount of *LktA*, mutants 11, 15 and 36 exhibited similar levels of *LktA* compared to the parent. These blots are not

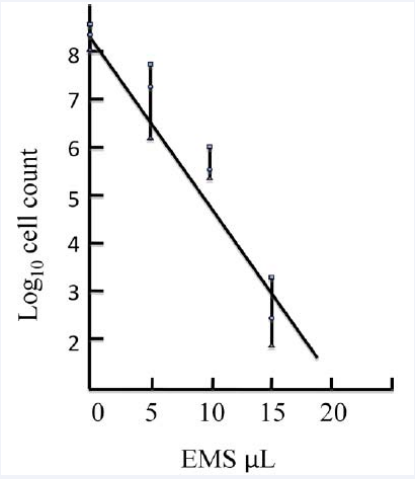

Figure 1 A kill curve of EMS on Mh. Results of three separate experiments, the bar showed data from each experiment and the average used for the plot.

Figure 2 Haemolytic vs non-haemolytic phenotype on blood agar plate. Mutants 15, 17, 34, 36 compared to parent SH1217.

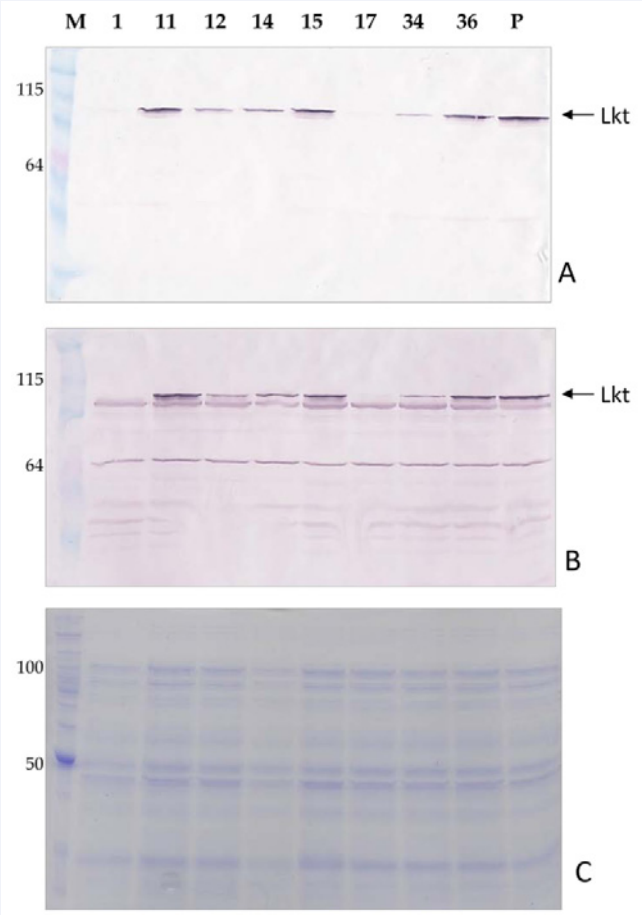

Figure 3 SDS-PAGE and Western immunoblot analysis of LktA in the non-haemolytic mutants. Total proteins were separated by SDS-PAGE and immunostained with antibodies against LktA. The results from triplicate gels were shown. Panel A: immunostained with monoclonal antibody 601. Panel B: immunostained with polyclonal antibody against LktA. Panel C: Coomassie Blue stained gel. The mutant numbers are as shown across the top, P refers to the parent SH1217. The molecular mass standards (in kDa) are shown on the left.

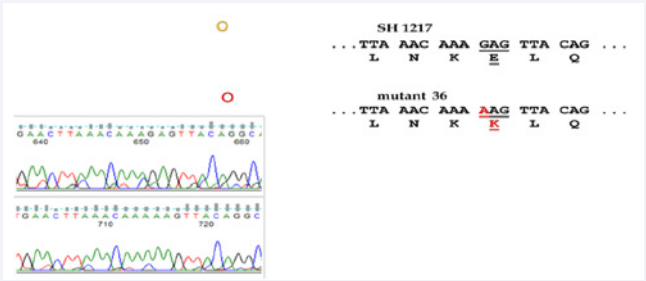

Figure 4 Nucleotide sequence of lktA from the parent SH1217 and mutant 36. The left panel showed the chromatograms from the sequence analysis. The right panel showed the nucleotide sequence over amino acid position 470 in LktA. The nucleotide change and the resulting amino acid substitution of glutamic acid to lysine in mutant 36 are shown in red.

quantitative and needs additional confirmation even though the results are reproducible. These mutants may have a defect in the *lktA* gene rendering it inactive.

Nucleotide sequence analysis

To determine if there is any mutation in the *lkt* promoter that could result in abolishing *lkt* expression, a 450 bp promoter region from seven mutants were amplified by PCR for sequence analysis. There was no alteration in these promoters (data not shown) suggesting the failure to produce Lkt is not due to a promoter mutation.

Since mutant 36 showed the presence of *LktA*, but a non-haemolytic phenotype, the 3 kbp *lktA* gene from this mutant was amplified for sequence analysis. The result in Figure (4) showed that this mutant has a G-C to A-T transition. This base substitution resulted in a codon change from GAG to AAG and glutamic acid to lysine (E470K) amino acid substitution at residue 470 in *LktA*. This amino acid position is highly conserved in other RTX toxins [Table 2]. Interestingly, amino acid position 470 in *Ap* ApxIII and *Aa* Lkt has a glutamine and a lysine residue, respectively, instead of glutamic acid. Neither of these toxins exhibit haemolytic activity suggesting that E470 may be important for toxin activity against erythrocytes.

CONCLUSION

EMS mutagenesis is a simple and effective means of creating base-substitution mutations. The result from sequence analysis of mutant 36 showing a G-C to A-T transition is in agreement with the preferred mutagenic activity of EMS [29,31,32]. The glutamic acid residue at position 470 in *LktA* is a highly conserved position in RTX with haemolytic activity suggesting that it could be important for toxin activity against erythrocytes and warrants further investigation. Several mutants do not produce *LktA* as shown by Western Immunoblot analysis. These are mutants which could harbour defect(s) that abolished *lkt* expression. They could reside in genes that code for regulators that control expression of the *lkt* operon. They are candidates for additional analysis to determine the location of the mutation and the loci/function affected. Further, any loci identified in these *Mh* mutants could have homologous loci in other RTX operons, giving it a wider implication on RTX regulation in these pathogens.

Table 1: Primer sequences for amplification of *lkt* promoter and *lktA* fragments.

Fragment 1, 780 bp product	
RT*lkt* C-For:	5'GGAAACATTACTTGGCTATGG
*lkt*227-Rev:	5' CGCTCAGTTAAGCCAATAGC
Fragment 2, 1.9 kbp product	
*lkt*227-F:	5' CAATCATTAACCCAAGCCGG
lktA-R:	5' TGAAGTCACTTCGTGTAGTGC
Fragment 3, 1 kbp product	
*lktA*2.5-F:	5' AGCCCGTGGAAACTATGGTGCT
*lktA*3.5-R:	5' GCCAACATAGTGAGGGCAACT

Table 2: Amino acid alignment of several RTX toxins over amino acid position 470. Shown are the six amino acids flaning position 470 illustrating the highly conserved glutamic acid residue and the lysine residue in mutant 36.

	470
Ec HlyA	Y N K E Y S
As AshA	L N K E L Q
Ap ApxI	Y N K E Y S
Ap ApxII	L N K E L Q
Ap ApxIII	F N K Q Y E
Aa Lkt	L R E K Y K
Mh LktA	L N K E L Q
mutant 36	L N K K L Q

Abbreviations: *Ec*: *E. coli*; *As*: *A. suis*; *Ap*: *A. pleuropneumoniae*; *Aa*: *A. actinomycetemcomitans*; *Mh*: *M. haemolytica*.

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