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Cloning and Expression of the *Pasteurella haemolytica* A1 leukotoxin Gene in *Bacillus subtilis*

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*Bacillus subtilis*을 이용한 *Pasteurella haemolytica* A1 leukotoxin 유전자의 cloning 및 발현

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초 록 : Bovine Pneumonic Pasteurellosis는 輸送熱로 일반적으로 알려져 있는 질병으로서, 여러가지 요인의 複合的인 작용에 의해 발병하는 것으로 알려져 있으나, *Pasteurella haemolytica* A1이 가장 主要한 因子로 밝혀져 있다. *P haemolytica* A1은 leukotoxin(LKT), lipopolysaccharide(LPS), capsular polysaccharide 등 여러가지의 病原性因子을 생성한다. 이들 인자중 LKT가 가장 중요한 병원성인자로 밝혀져 있다. 이에 본 실험은 *P haemolytica* A1의 LKT 유전자를 *Bacillus subtilis*에서 發現시킴으로서 LPS에 汚染되지 않은 LKT을 대량으로 생산할 목적으로 실시되었다. 실험의 첫 段階로서 pLKT52 plasmid을 *Sau3* A1의 제한효소을 이용하여 部分消化시킨 후 이 부분 消化된 유전자들로부터 3~5kb 크기의 유전자들을 순수분리하여 pUC18와 결합시킨 후 *E coli* NM522에 形質轉換시켰다. 이때 형질전환된 균주들은 LKT에 대한 단크론 항체인 MAb601을 이용하여 colony blot 법에 의해서 LKT 유전자 보유 및 發現與否을 조사하였다. 이들 양성 clone들은 制限酵素分析, 鹽基序列分析 및 Western blot 등에 의해서 再確認하였다. 총 9개의 양성 clone중 위의 방법에 의해서 한 clone을 選擇하여 *lkt*CA insert를 재분리하여 shuttle vector에 subcloning 하였다. Subcloning된 LKT 유전자들은 shuttle vector의 種類(pHPS9, p602/20, pHPS9-*Sac*)와 各其 다른 種類의 *B subtilis*(spoO12A, BR121, WB30, Raj1105) 宿主內에서 발현정도를 Western blot 법에 의해서 比較하였다. 이때 最適發現條件은 p602/20와 pBL1의 dual plasmid system을 이용하여 *B subtilis* spoO12A에서 2시간동안 IPTG로 발현을 誘導하는 것이었다. *B subtilis*에서 발현된 LKT을 visual 법과 neutral red uptake 법을 이용하여 소 肺

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胞 大食求에 대한 biological activity 를확인하였다. 발현된 LKT에 대한 LPS 오염은 LKT을 SDS-PAGE 후 silver stain에 의해서 확인하였다. 본 실험을 통해서 볼 때에 *lkt*CA 유전자를 保有하고 있는 p602/20는 *B subtilis*에서 매우 不安定하였고, 발현된 LKT는 細菌自體에서 생성되는 protease들에 의해서 破壞됨으로서 濃度가 매우 낮았다. 이러한 문제점들은 다음 段階의 실험에서 해결되어야할 문제들이다.

Introduction

Shipping fever or bovine pneumonic pasteurellosis is the disease that cause the greatest economic loss in the North American cattle industry[1,2]. It is believed to be caused by the interaction of stressful management practices, viruses and bacteria. The primary bacterial agent that is responsible for the clinical disease and pathophysiologic events leading to lobar fibrinonecrotizing pneumonia is *Pasteurella haemolytica* biotype A, serotype1 (A1)[3-5].

P haemolytica A1 elaborates several potential virulence factors including lipopolysaccharide(LPS) with endotoxin properties, an exotoxic leukotoxin (LKT), capsular polysaccharide, fimbriae, and exocellular enzymes[6,7]. Of these virulence factors, the LKT and LPS have received the most attention. The ease with which the LPS can be purified and its availability has allowed us to delineate its role in the pathogenesis of lung injury in pneumonic pasteurellosis[8-12]. In contrast, very little is known about the biochemical nature, the mode of action and its contribution to lung injury because of the difficulties in obtaining purified LKT.

LKT is one of a family of Repeats in ToXin(RTX) which is a pore-forming cytolysin which possess similar functional and antigenic properties to other Ca^{++} pore-forming cytolysins produced by *Escherichia coli, Actinobacillus, Proteus, Morganella* and *Bordetella pertussis*[13,14]. Therefore, it is possible that the LKT-created transmembrane pores on leukocytes and vascular endothelium, will allow Ca^{++} influx into these cells[15,16]. The increase in the concentration of intracellular Ca^{++} can lead to activation of alveolar macrophages(AMs) and other leukocytes[17], resulting in the production of inflammatory cytokines and injurious lipid mediators in the lung[16]. The LKT may also contribute to the inflammatory response by stimulating a respiratory burst and degranulation of lysosomes in PMNs and AMs in the lung[18,19], thus augmenting the release of injurious enzymes and toxic oxygen radicals[19]. Surprisingly, the LKT has a narrow target cell specificity being cytocidal only to ruminant leukocytes and platelets[20,21]. However, to this end, we have not still identified the unique binding receptor(s) for LKT on ruminant leukocytes.

The gene cluster encoding synthesis, activation and secretion of LKT has previously been cloned, sequenced and expressed in *E coli*[22-25]. The LKT gene cluster(*lkt* CABD) consists of 4 genes, the C gene which is required for activation of LKT, the A structural gene which encodes synthesis of the LKT product itself, and B and D genes which are required for secretion of LKT in *P haemolytica* and *E coli*[22,24]. Attempts to purify the recombinant LKT(rLKT) from other components of *E coli* have been unsuccessful because it is tightly complexed with LPS, a component notoriously difficult to remove.

We propose that production of the LKT in a Gram-positive bacteria would eliminate this problem of LPS contamination. *Bacillus subtilis* undoubtedly represents a particularly interesting host for genetic engineering because it is apathogenic, and has the ability to express and synthesize large amounts of the heterologous proteins. There have been several published reports on the use of *B subtilis* as a surrogate host for expressing genes from Gram-negative bacteria to avoid LPS contamination[26]. Also, the bacteria is extensively used in the microbial engineering industry since it can secrete proteins into the

culture supernatant[26,27]. In medical research[28~30], *B subtilis* has been used as an expression host to produce pure subunit immunogens to avoid LPS or other exotoxin contaminants which can induce secondary reaction in the host. However, several disadvantages such as low transformation frequency and instability of plasmid in direct cloning and expression have been encountered in the cloning and expression of genes in *B subtilis*[31,32]. In order to overcome these problems, shuttle vectors have been used to clone and express a gene in *B subtilis*. Therefore, in these experiments, we subcloned and expressed the *lkt*CA genes in *B subtilis* using shuttle vectors.

Materials and Methods

Bacterial strains, media and plasmids : *Escherichia coli* DH5α, and NM522, and *Bacillus subtilis* spoO12A (spo-), BR121(spo+), Raj1105[33] and WB30[34] obtained from Dr Anderson's laboratory, Department of Oral Sciences, University of Minnesota were used as host cells for cloning, subcloning and expression of *P haemolytica* leukotoxin gene in *E coli* and *B subtilis*, respectively. Luria-Bertani(LB) medium(1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl) and DM3 medium (500ml of 1M sodium succinate (pH 7.3), 100ml of 5% casamino acids, 50ml of 10% yeast extract, 100ml of 3.5% K_2HPO_4 and 1.5% KH_2PO_4, 25ml of 20% glucose, 20ml of 1M $MgCl_2$ and 5ml of 2% bovine serum albumin (BSA) per 1,000ml of distilled water) were used for propagating *E coli* and *B subtilis*, respectively. Plasmids, pLKT52[35], pSM147(ATCC53040), pUC18, pBluescript KS(+), pHPS9[33], p602/20 and pBL1[36] and sucrose-inducible (*Sac*) promoter[34], were used for cloning, subcloning and expression in *E coli* and *B subtilis*. pLKT52 carrying the *lkt*CABD gene cluster was obtained from Reggie Lo, University of Guelph, Canada and the rest of the plasmids were obtained from Dr Anderson's Laboratory, University of Minnesota, USA.

Monoclonal antibody : LKT-neutralizing monoclonal antibody(MAb601) was used for immunoscreening and Western blot. The generation and characterization of the MAb601 was described by Gentry and Srikumaran [37].

Nucleotide sequencing : pBY1 which is a pBluescriptKS(+) carrying *lkt*CA genes, was used for sequencing of the cloned *lkt*A gene. Sequencing was done by the fluorescent DNA sequencing method adapted from Sanger's dideoxy terminator sequencing using the PRISM™ ready reaction dyedeoxy™ terminator cycle sequencing kit (Applied Biosynthesis) with forward and reverse M13 universal primers. The sequencing was done at the Microchemical Facility, Institute of Human Genetics, University of Minnesota, USA.

Cloning of *lkt* CA genes : Procedure for subcloning of *lkt* CA genes is summarized in Fig 1. All restriction endonucleases used in the subcloning were purchased from Gibco/BRL(Grand Island NY).

1) Cloning of *lkt* CA genes into pUC18 and immunoscreening : The pLKT52 plasmid DNA carrying the *lkt*CABD insert was purified from *E coli* and partially digested with *Sau*3A1 in order to excise the *lkt*CA genes from the rest of the *lkt*CABD gene cluster. The partially digested DNA was resolved by electrophoresis in 1% agarose gel and 3~5kbp DNA fragments were recovered from the gel using the GenecleanII *kit* (Bio1101, La Jolla, Calif). These DNA fragments were ligated with T4 DNA ligase to the plasmid vector pUC18 which was digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase. The pUC18 construct containing the *lkt*CA insert was transformed into *E coli* NM522 by the $CaCl_2$ method[38] and plated onto LB plates containing 50μg of ampicillin(AP) per ml and 1mM isopropyllthio-β-galactoside(IPTG, Gibco/BRL). Replica membranes were obtained by colony transfer from the master membrane. Colonies on the replica membranes were immunoscreened for identifying clones expressing LKT with monoclonal antibody MAb601 using a previously described procedure with some modifications[39]. Briefly, replicate membrane preparations containing transformant colonies were lysed by chloroform vapor for 15min and then treated with a buffer containing 50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM $MgCl_2$, 5% non-fat dried milk(NFDM), 2μg of DNase, and 40μg of lysozyme per

ml overnight at room temperature with shaking. Membranes were rinsed with Tris-saline(50mM Tris-HCl(pH 7.5), 150mM NaCl) and 5% NFDM to remove any remnants of colonies. The membranes were reacted with a 1 : 2,000 dilution of MAb601 at 37℃ for 2h and rinsed for 10 min four times with Tris-saline-5% NFDM-0.05% Nonident P-40(Sigma). The membranes were incubated with a 1 : 1,000 dilution of horseradish peroxidase conjugated anti-mouse goat IgG(Cappel) at 37℃ for 1h. After washing the membrane for 10 min four times with Tris-saline with 5% NFDM, color was developed in 0.06% (w/v) 4-chloro-1-naphthol and 0.06%(v/v) hydrogen peroxide. Positive colonies were identified by a purple color. Positively reacting clones were recovered from the replica plates and then rescreened to confirm LKT production.

2) **Characterization of the cloned insert** : Positive clones identified by immunoscreening were subcloned and rescreened to verify clonality. Insert size and restriction map of insert were determined by restriction endonuclease digestion and agarose gel electrophoresis with ethidium bromide staining. Approximately 400~450bp at both 5' and 3' end of the cloned insert were sequenced to verify that the cloned insert was indeed the *lkt*CA genes. The results were compared with previously published data[24,25] to confirm the presence of the *lkt*CA. The expression of cloned *lkt*CA genes carried in pUC18 in the presence of IPTG was detected by Western blot[40]. These results led us to select one recombinant from pUC 18 containing the *lkt*CA insert, designated pUY8 for further studies.

3) **Subcloning of the *lkt*CA genes into shuttle vector** : pUY8, a construct carrying the *lkt*CA genes and confirmed as described above, was digested with *Eco*RI and *Hind*III and subjected to agarose gel electrophoresis in TAE buffer(0.04M Tris-acetate, 0.001M EDTA). The 4kb *lkt*CA insert was identified and purified using the GenecleanII kit. The 602/20, shuttle expression vector, was subjected to double digestion with *Eco*RI and *Hind*III and ligated with the *lkt*CA insert. The ligated plasmid was transformed into *E coli* DH5α and plated on LB medium containing 15μg of kanamycin per ml. Transformants were selected, screenol, and the presence of *lkt*CA genes was confirmed after digestion with restriction enzymes. Also, the 4kb insert was ligated with pBluescript KS(+) digested with *Eco*Rl and *Hind*III and selected recombinant plasmids were selected by same method described above. The plasmid, designated as pBY 1, was used for sequencing.

4) **Transformation of the shuttle vectors into *B subtilis*** : The plasmid p602/20 carrying the 4kb *lkt*CA fragment, referred to as pBY2, was transformed into *B subtilis* spoO12A harboring pBL1 by protoplast transformation[26]. Briefly, *B subtilis* was grown in 15ml of medium 416 for 12h at 37℃ with shaking, and harvested by centrifugation at 5,000×g for 5 min. The cells were resuspended in 0.5ml of SMMP/BSA [mixture of equal volume of 2×SMM buffer (1.4M sucrose, 0.04M maleic acid and 0.04M $MgCl_2$(pH 6.5) and 4×penassay broth with 1% BSA] with sterile lysozyme (final concentration ; 2mg/ml) and incubated at 37℃ for 15~30 min with gentle shaking to generate protoplast. Protoplasts were harvested by centrifugation at 5,000×g for 5min and resuspended in 2ml of SMMP/BSA. Plasmid DNA suspended in TE (0.5~1.0g) was mixed with 250μl of SMMP/BSA solution, 250μl of protoplast suspension, 1.5ml of 40% PEG 8000 solution was incubated for 2min at room temperature. Thereafter, 5ml of SMMP/BSA solution was added and mixed gently by pipetting up and down 2~3 times. The protoplast reacted with DNA was collected by centrifugation at 5,000×g for 5min and resuspended in 1ml of SMMP/BSA solution. The solution was incubated with gentle shaking at 37℃ for 2h for phenotypic expression of drug resistant genes. After the incubation, the solution was diluted in appropriate amounts of SMMP/BSA solution, plated on DM3 medium containing 10μg of erythromycin and 100μg of kanamycin per ml and incubated at 37℃ for 1~3 days. Several transformants were picked, plasmids recovered, and the presence of *lkt*CA genes was confirmed after digestion with restriction enzymes.

Expression of the subcloned *lkt*CA genes in *B subtilis*.

1) Expression of the subcloned *lkt*CA genes in *B subtilis* : *B subtilis* spo012A harboring both pBY2/pBL1 was propagated until it reached mid logarithmic phase in 5ml of medium 416 using the following method : the *B subtilis* was first cultured overnight in 2ml of medium 416 at 30℃ with shaking. The culture was then diluted into 5ml of the same medium at a 1 : 10 ratio and grown at 30℃ with shaking until it reached mid logarithmic phase (Klett value at A600=1.0). Production of rLKT in *B subtilis* harboring pBY2 and pBL1 was induced by adding IPTG (from stock solution of 200mg/ml) to a final concentration of 400μg/ml and growing the culture in a shaking platform at 30℃ for 3 to 4h. After IPTG induction, the bacterial cells were harvested by centrifugation, suspended in 500μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) sample buffer and then incubated in boiling water for 10 min. To determine whether the rLKT expressed in *B subtilis* was secreted out, culture supernatant of the *B subtilis* harboring pBY2 and pBL1 and induced by IPTG was harvested, freeze-dried and suspended in 500μl of SDS-PAGE buffer. The samples were subjected to SDS-PAGE using 10% acrylamide gel. For the *Sac* promoter, *B subtilis* harboring pBY3 was grown in 416 medium in the presence of 2% sucrose as described in individual experiments and expression of rLKT with the promoter was analyzed by a method described earlier.

2) Confirmation of the presence of the rLKT expressed in *B subtilis* by SDS-PAGE and Western blot : Cell extracts from *E coli* transformants or cell-extracts from *B subtilis* transformant clones were analyzed for the presence of rLKT by SDS-PAGE followed by Western blot[40], using MAb601 as the probe. The preparation and running of SDS-PAGE was done as described by Laemmli[41]. Pre-stained *Mr* markers(Bio-rad) were applied to the SDS-polyacrylamide gel for reference, following transfer to nitrocellulose membrane. One hundred μl of each sample was analyzed on 10% SDS-PAGE gel and electrophoresis was carried out in Tris-glycine buffer (25mM Tris base, 250mM glycine, 0.1% SDS) at 10mA for 1hr and 15mA for 4hrs. After SDS-PAGE, the gels were either stained with Coomassie Brilliant Blue R-250 solution or electrophoretically transferred onto nitrocellulose membrane(Immobilin-P, Millipore) in the electrode solution (20mM Tris base, 150mM glycine, and 20%(v/v) methanol). The membrane was probed with a 1 : 2,000 dilution of the leukotoxin-neutralizing antibody MAb601. The LKT-specific band was detected by incubation with horseradish peroxidase-conjugated goat-anti-mouse immunoglobulinG (Cappel) followed by incubation with a substrate solution containing 0.06% (w/v) 4-chloro-1-naphthol and 0.06%(v/v) H_2O_2 as the substrate.

3) Comparison of expression of rLKT with different shuttle vectors and *B subtilis* host strains : In order to determine the optimal expression of LKT in *B subtilis*, we used three different shuttle vectors, pHPS9, p602/20 and pBL1, and pHPS9-*Sac* and four different host strains, spoO12A, BR121, WB30 and Raj1105. To maximize the expression of LKT in *B subtilis*, overnight culture, IPTG induction for 2h and sucrose induction overnight were used for pHPS9, p602/20 and pBL1 and pHPS9-*Sac*, respectively. When using pHPS9, p602/20 with pBL1, *B subtilis* spoO12A and BR121 strains were used as host bacteria. Strains WB30 and Raj1105 of *B subtilis* were used as hosts with pHPS9 carrying the *Sac* promoter. In order to compare the amount of expression, the concentration of expression bacteria was adjusted to the same density after induction, and subjected to SDS-PAGE followed by Western blot analysis.

Evaluation of biological activity of the rLKT expressed in *B subtilis* : The biological activity of the rLKT on bovine alveolar macrophages(BAMs) was determined by visual assay and neutral red uptake assay as previously described[23,24]. To detect biological activity of the rLKT expressed in *B subtilis*, cellular lysates were prepared from *B subtilis* spo012A(spo-) harboring either pBY2 and pBL1, or p602/20 and pBL1, by disrupting the protoplast which was obtained by incubating the *B subtilis* harboring either pBY2 and pBL1 or native plasmid p602/20 and pBL1 with lysozyme(final concentration-5μg/ml) in SMMP-BSA solution at 37℃ for 30min by passing through a French pressure cell at 10,

000psi three times. Disrupture of the protoplast was confirmed under a microscope. The protein concentration was measured using the standard protein assay kit (Boeringer Manheim).

1) Visual assay : The standard visual assay was carried out as previously described by Change et al[23]. Two-fold dilutions of the cellular lysates of *B subtilis* harboring either pBY2 and pBL1, or p602/20 and pBL1 were incubated with 10^6 BAMs suspended in 1ml of Leibovitz L-15 medium(Gibco/BRL) containing L-glutamine and 10% fetal calf serum, for 1h at 37℃. Crude LKT was prepared from culture supernatant of *P haemolytica* 12296 and then used as positive control. At the end of the incubation period, the BAMs were visually examined for evidence of cytolysis using a phase-contrast microscope.

2) Neutral red uptake assay : The neutral red uptake assay with BAMs was performed using the method described by Greer et al[42]. Two hundred μl of 1.2×10^6 viable BAMs/ml were seeded in a 96-well flat-bottomed tissue culture plate. After centrifugation at 500×g for 10 min at room temperature(RT), the overlaying medium was removed by rapid inversion of the plate and 200μl of a two-fold diluted cellular lysates collected from *B subtilis* harboring either pBY2 and pBL1, or p602/20 and pBL1 were added to appropriate wells and allowed to incubate with the cells for 60min at 37℃ inside a 5% CO_2 incubator. After centrifugation (500×g, 10 min. RT), the supernate were decanted and wells were washed with 200μl of saline. After washing with saline, 100μl of a neutral red working solution($O.D_{570}$=1.7) was added to each well and allowed to incubate for a further 60 min at 37℃ inside the same incubator. After centrifugation of the plate (550×g, 10 min. RT), the dye solution was discarded and wells were washed twice with saline. Lysis of the cells with concomitant release of the dye was accomplished by the addition to each well of 200μl of 0.05M acetic acid-0.5% SDS solution. The plate was covered and the resultant dye concentration was determined the next day by measuring O.D. at 570nm with an ELISA reader. The leukotoxic activity was determined from the mean of triplicate wells using the formula : (O. D. of 100% viable cell control wells - O. D. of test wells) / O. D. of 100% viable cell control wells×100. Validation that the biological activity of rLKT on alveolar macrophages was due to specific cytotoxicity, was determined by the capacity of murine ascites fluid containing LKT-neutralizing antibody (MAb601) to inhibit this bioactivity[43].

Detection of lipopolysaccharide(LPS) in the lysates of *B subtilis 12A* : The presence of LPS in the cellular lysates of *B subtilis* harboring either pBY2 and pBL1, or p602/20 and pBL1, *E coli* harboring pBY2, and crude LKT prepared from *P haemolytica* culture supernatant was detected by silver staining[44] followed by Coomassie Brilliant Blue R-250 stain. Glassware and electrophoresis plates used for silver staining were soaked in 1% E-toxaclean(Sigma) solution overnight and rinsed thoroughly first with tap water and then with depyrogenated water. A clean glass dish was used for each step. After electrophoresis, the LPS band was fixed by soaking the gel in 200ml of 25%(v/v) isopropanol in 7%(v/v) acetic acid overnight. Oxidation was performed by incubation of the gel in 150ml of distilled water(DW) with 1.05g of periodic acid and 4ml of 25% isopropanol in 7% acetic acid for 5 min. After oxidation, the gel was washed eight times, 30 min each time with 200ml of DW. The gel was silver stained for 10 min in a solution consisting of 0.1N NaOH (28ml), concentrated(29.4%) ammonium hydroxide (1ml), 20% silver nitrate (5ml) and DW (115ml) and then the gel was washed four times each time with 200ml of DW and then the gel was exposed to developing solution (50mg of citric acid and 0.5ml of 37% formaldehyde in 1 liter of D. W.) for 10~20 min. The reaction was terminated using a stop solution (200 ml of DW and 10ml of 7% acetic acid).

Results

Cloning of *lkt*CA genes into shuttle vector : The strategies used to construct recombinant are depicted in Fig 1. All recombinant plasmids constructed from this experiment and vectors used in this experiment are summarized in Table 1. In order to clone the leukotoxin genes (*lkt*CA) into a shuttle vector, first of all, pLKT52

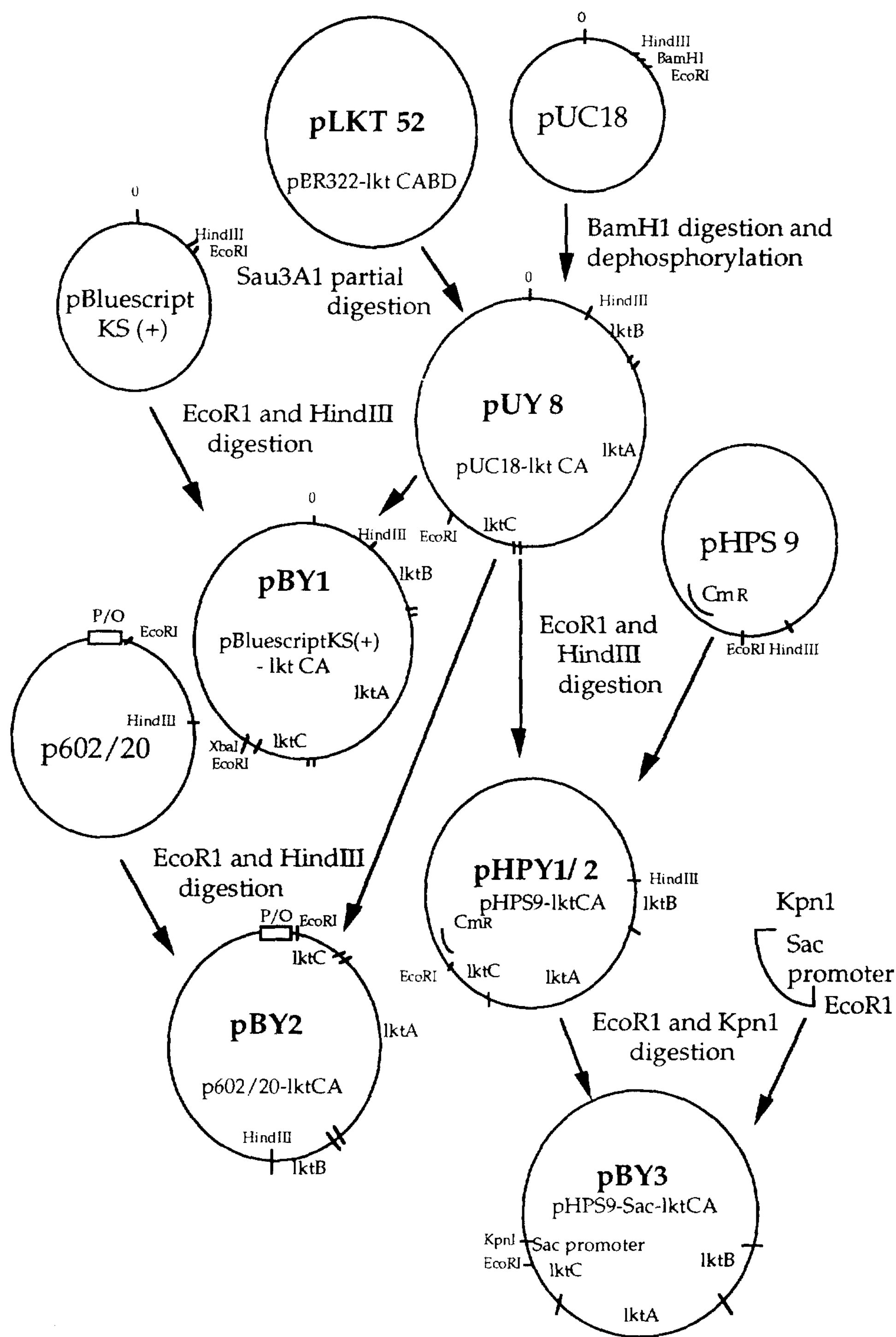

Fig 1. Strategy for construction of shuttle plasmids to express *Pasteurella haemolytica lkt* CA genes in *Bacillus subtilis*.

Table 1. Shuttle vectors and recombinant plasmids carrying *Pasteurella haemolytica* leukotoxin (*lkt* CA) genes

In *Escherichia coli*

Recombinant Plasmids	Original Vector	Origin of Insert	Insert size (Kb)	Western blot with MAb 601 probe
pTR 52	pSM 147	pLKT 52[a]	4	++
pUY 1	pUC 18	pLKT 52[a]	2.8	+
pUY 2	pUC 18	pLKT 52[a]	1.5	+
pUY 3	pUC 18	pLKT 52[a]	9.6	++
pUY 4	pUC 18	pLKT 52[a]	4.5	+
pUY 5	pUC 18	pLKT 52[a]	8.0	+++
pUY 6	pUC 18	pLKT 52[a]	11	+++
pUY 7	pUC 18	pLKT 52[a]	6	+
pUY 8	pUC 18	pLKT 52[a]	4	+
pUY 9	pUC 18	pLKT 52[a]	3,7	++
pHPY 1	pHS 9	pUY 8[b]	4	++
pHPY 2	pHS 9	pUY 8[b]	4	++
pBY 1	pBluescript KS(+)	pUY 8[b]	4	++
pBY 2	p602/20	pUY 8[b]	4	-
pBY 2+pBL1	p602/20,pBL1	pUY 8[b]	4	-

[a] partial digests of pLKT 52 with *Sau*3A1 [b] digested by *Hin*dIII and *Eco*RI

In *Bacillus subtilis*

Recombinant Plasmids	Original Vector	Origin of Insert	Insert size (Kb)	Western blot with MAb 601 probe
pHPY1[c]	pHPS 9	pUY 8	4	+
pHPY2[d]	pHPS 9	pUY 8	4	+
pBY2[c]	p602/20[f]	pUY 8	4	+++
+pBL1	pBL 1			
pBY3	pHPS 9	pHPY1	4	+
	Sac promoter			

[c, d, e] ; originated from *E. coli* plasmid pHPY1, pHPY2, pBY2, respectively

[f] ; dual-plasmid repressible system for *Bacillus subtilis.*

carrying the *lkt* CABD gene cluster was partially digested with *Sau*3A1 and subjected to 1% agarose gel. Three to five kb fragments were purified from the gel, ligated with *Bam*HI-digested and dephosphorylated pUC18 and transformed into *E coli* NM522. Out of 1,000 transformants, nine positive clones, named pUY1 to pUY9, were identified from pUC18 constructs by immunoscreening with MAb601 as the probe(Table 1). Of the nine positive clones generated from pUC18 constructs, pUY8 was selected for further studies after confirmed by restriction enzyme analysis and Western blot with MAb601 as the probe. Four kb *lkt*CA insert was purified from pUY8 by *Eco*RI and *Hind*III digestion, ligated with pBluescript KS(+) and transformed into *E coli* DH5α. pBY1 was generated from the ligation. This clone was used for sequencing to confirm the insert as *lkt*CA genes. The cloned *lkt*CA genes were from 3' end of *lkt*C to 5' end of *lkt*B of the *lkt*CABD gene cluster (345~4281). The insert confirmed as leukotoxin genes was purified from the clone pBY1, ligated with shuttle vectors, pHPS9 or p602/20 and transformed into *E coli* DH5α. pHPY1 and pHPY2 and pBY2 were generated from shuttle vector, pHPS9 and p602/20, respectively. pHPY1, pHPY2 and pBY2 were transformed into *B subtilis* spoO12A by protoplast transformation. In order to increase the expression efficiency of the insert, pBY3 was generated by insertion of *Sac* promoter which was the sucrose inducible promoter from *gro*ESL gene of *B subtilis*, at just front of the insert(*lkt*CA) in pHPY1. The pBY3 was expressed by *B subtilis* in the presence of 2% sucrose. Also, pTR52 was constructed from another shuttle vector, pSM147 with 3~5kb fragments purified from partially digested pLKT 52 with *Sau*3A1. The pTR52 was positive in colony immunoblot and Western blot with MAb601 in *E coli*, but it could not be transformed into *B subtilis* using protoplast transformation and electroporation.

Expression of the subcloned *lkt*CA genes in *E coli* and *B subtilis* : Expression of *lkt*CA genes in *E coli* and *B subtilis* were detected by immunoscreening and Western blot with MAb601 as the probe. In expression of cloned leukotoxin genes in *E coli*, pUY2 which was positive in colony immunoblot, pBY2, and pBY2 and pBL1 plasmids were negative and did not react with MAb601 in Western blot (Table 1 and Fig 2). All recombinant plasmids generated with shuttle vectors in *B subtilis* reacted with MAb601 in Western blot (Table 1 and Fig 3). Also, culture supernatant from *B subtilis* harboring both pBY2 and pBL1 also showed immunoreactivity with MAb601 in Western blot(lane 5 in Fig 3).

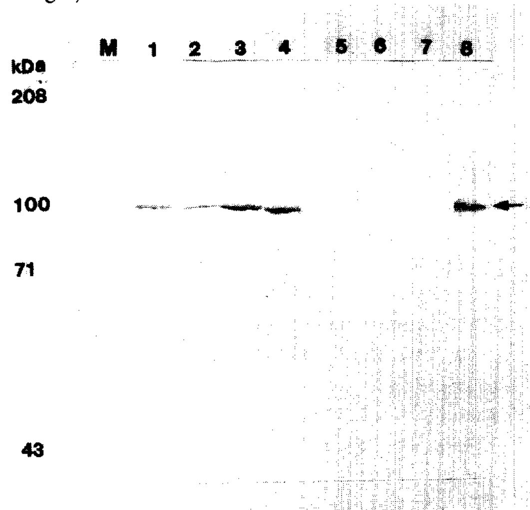

Fig 2. Immunoblot analysis for detection of *P haemolytica* LKT expressed in the cell lysates from *E coli* DH5α harboring different recombinant plasmids constructed from various plasmid vectors.
Lanes : M, molecular weight markers ; 1, pTR52 (pSM147) ; 2, pUY8 (pUC18) ; 3, pBY1 (pBluescript KS(+)) ; 4, pHPY1 (pHPS9) ; 5, pBY2 (p602/20) ; 6, pBY2 and pBL1 ; 7, pUC18 (vector only) ; and 8, crude LKT prepared from culture supernatant of *P haemolytica* A1. Electrophoretically transferred proteins from SDS-PAGE gel onto nitrocellulose membrane was reacted with a 1:2,000 dilution of the leukotoxin-neutralizing MAb601. The blot was developed, followed by using a horseradish peroxidase-conjugated goat-anti-mouse immunoglobulin G (Cappel) by using 4-chloro-1-naphthol and H_2O_2 as the substrate. Arrow indicates the LKT bands.

1) Kinetics of expression of *P haemolytica* LKT in *B subtilis* : In order to determine the kinetics of the LKT expression in *B subtilis, B subtilis* spoO12A harboring both pBY2 and pBL1 was cultured and induced with IPTG as described in Materials and Methods. LKT-spec-

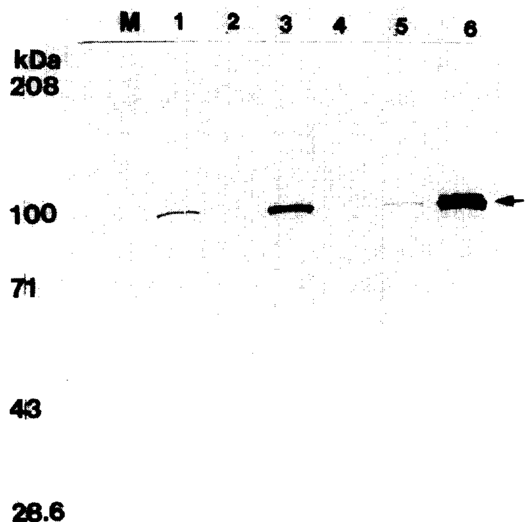

Fig 3. Immunoblot analysis for detection of *P haemolytica* LKT expressed in the cell lysates from *B subtilis* harboring different recombinant plasmids constructed from shuttle vectors or culture supernatant from *B subtilis* harboring pBY2 and pBL1.
Lanes : M, molecular weight markers ; 1, pHPY1 ; 2, pHPS9 ; 3, pBY2 and pBL1 ; 4, p602/20 and pBL1 ; and 5, culture supernatant of *B subtilis* harboring pBY2 and pBL1, and 6, crude LKT prepared from culture supernatant of *P haemolytica* A1. Processing of the blot was similar to that described in Fig 2. Arrow indicates the LKT bands.

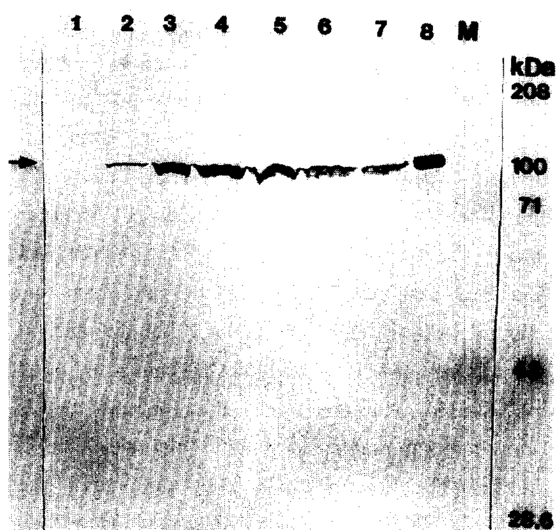

Fig 4. Kinetics of expression of *P haemolytica* LKT in *B subtilis* spoO12A harboring pBY2 and pBL1 following induction with IPTG. Lane 1, *B subtilis* harboring p602/20 and pBL1 ; Lanes 2, 3, 4, 5, and 6 - 0, 1, 2, 3, and 4hr culture of *B subtilis* harboring pBY2 and pBL1 after IPTG induction ; lane 7, 4 hr culture of pBY2 and pBL1 without IPTG induction ; lane 8, crude Lkt prepared from culture supernatant of *P haemolytica* A1 ; and M. molecular weight markers. Processing of the blot in panel B was similar to that described in Fig 2. Arrows indicate the LKT bands.

ific bands were detected in cellular lysates of *B subtilis* spoO12A harboring pBY2 and pBL1 by SDS-PAGE analysis. There was no reactivity in native plasmid vector control(p602/20 and pBL1) with MAb601(lane 1 in Fig 4). The highest expression of LKT in *B subtilis* was shown at 2h after IPTG induction, after which the expression decreased gradually(Fig 4). However, LKT was expressed at 0h after IPTG induction(lane 2 in Fig 4) and even without IPTG induction(lane 7 in Fig 4).

2) Comparison of expression of *P haemolytica* LKT in *B subtilis.* : *B subtilis* 12A, Raj1105 and WB30 strains were used as hosts to select an appropriate host for the maximal expression of LKT. Recombinant plasmid pBY3 which carried the sucrose-inducible(*Sac*) promoter was used to express the LKT in the three strains of *B subtilis*. Comparison of expression of LKT by the three different strains of *B subtilis* in the presence or absence of inducer 2% sucrose at different times revealed that *B subtilis* Raj 1105 harboring pBY3 grown overnight in the presence of 2% sucrose had the highest expression of LKT(data not shown). The expression of LKT in the cellular lysate of *B subtilis* spoO12A harboring pHPS9(vector control) was not detected. Comparison of LKT expression by pBY2 and pBL1 transformed into *B subtilis* spoO12A with those with pBY3 transformed into *B subtilis* spoO12A, Raj1105 and WB30, expression of LKT by pBY2 and pBL1 in *B subtilis* spoO12A was shown to be higher than that of pBY3 transformed into other *B subtilis* strains(data not shown). Furthermore, comparison of the expression of LKT in *E coli* and *B subtilis, B subtilis* harboring pBY2 and pBL1 showed higher expression than the expression in *E coli* DH5α harboring pTR52 when compared by Western blot analysis (data not shown). Spo- strain, *B subtilis* spoO12A, showed higher expression than spo^{+} strain, *B subtilis* BR121 (data not shown).

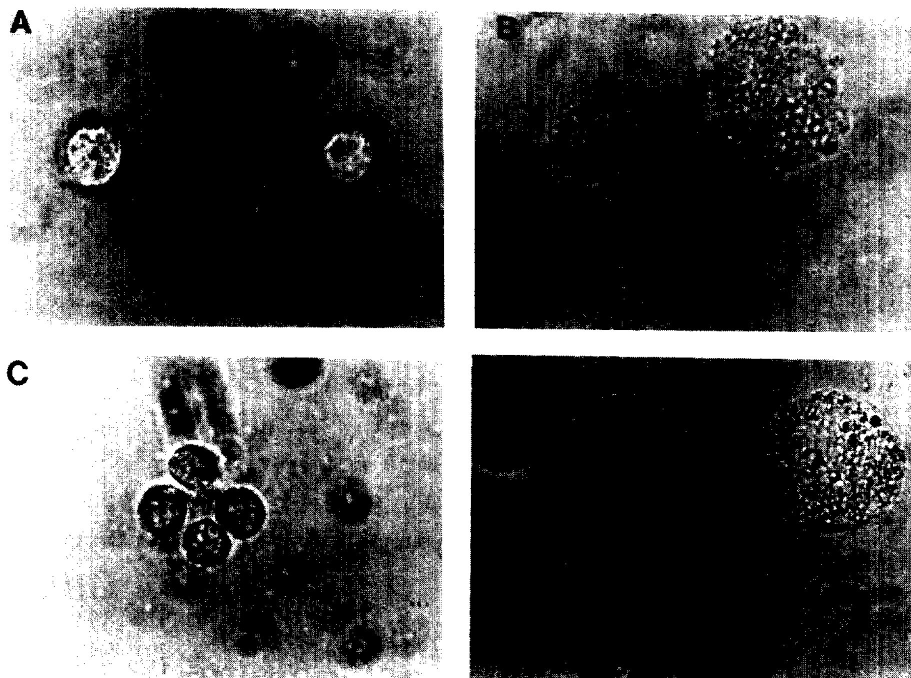

Fig 5. Leukotoxic activity of recombinant LKT expressed in *B subtilis* harboring pBY2 and pBL1 on bovine alveolar macrophages (BAMs). BAMs were exposed to lysates from *B subtilis* for 1 h and examined under a phase-contrast microscope. A-normal BAMs, B-BAMs exposed to crude LKT from *P haemolytica* A1 ; C-BAMs exposed to lysates from *B subtilis* harboring p602/20 and pBL1 ; D-BAMs exposed to lysates of *B subtilis* harboring pBY2 and pBL1.

Evaluation of biological activity of the rLKT expressed in *B subtilis* : Biological activity of the rLKT expressed in *B subtilis* was measured by the visual assay and neutral red uptake assay using cellular lysates of *B subtilis* harboring both pBY2 and pBL1 after IPTG induction. In the visual assay, BAMs exposed to cellular lysate of *B subtilis* harboring pBY2 and pBL1 (Fig 5D) showed cytotoxic changes such as cell swelling which was similar to that observed with BAMs exposed to crude LKT (Fig 5B), when examined by a phase-contrast microscope. However, there was no morphological change observed in BAMs exposed to cellular lysate of *B subtilis* harboring both native plasmid p602/20 and pBL1 (Fig 5C). In the neutral red uptake assay to compare cytotoxic activities of *B subtilis* harboring pBY2 and pBL1 with p602/20 and pBL1, the highest cytotoxic activity obtained with cellular lysates of *B subtilis* harboring pBY2 and pBL1 was 31.3±4.24 %. By contrast, the cytotoxic activity of cellular lysates of *B subtilis* harboring p602/20 and pBL1(vector control) was always lower than 5% (Fig. 6) and monoclonal antibodies (MAb601) at optimal concentration (1:100 dilution) inhibited completely this cytotoxic activity.

Detection of LPS contamination : The presence or absence of LPS in cellular lysates from *B subtilis* harboring (pBY2 and pBL1) and (p602/20 and pBL1), cellular lysate of *E coli* harboring pBY8, and crude LKT were evaluated on SDS-PAGE gels subjected to silver staining, followed by Coomassie Brilliant Blue R-250 staining. As shown in Fig 7, LPS was not detected in cellular lysates from *B subtilis* harboring either pBY2 and pBL1 or p602/20 and pBL1(lanes 1 and 2) while LPS was detected in cellular lysates from *E coli* and crude LKT from culture supernatant of *P haemolytica*(lanes 4 and 5). Also, no LPS was detected in all reagents used in this staining(lane 3). However, it should be noted that no attempts were made to detect any endotoxin from cellular lysates using

the chromogenic *Limulus* amebocyte assay.

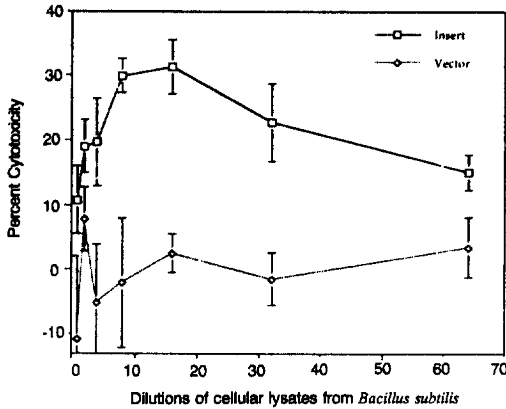

Fig 6. Leukotoxic activity of recombinant LKT expressed in *B subtilis* harboring pBY2 and pBL1 after IPTG induction, was evaluated using bovine alveolar macrophages (BAMs) as target cells in an automated neutral red uptake assay. BAMs were exposed to lysates from *B subtilis* harboring either pBY2 and pBL1 (Insert) or p602/20 and pBL1 (Vector).

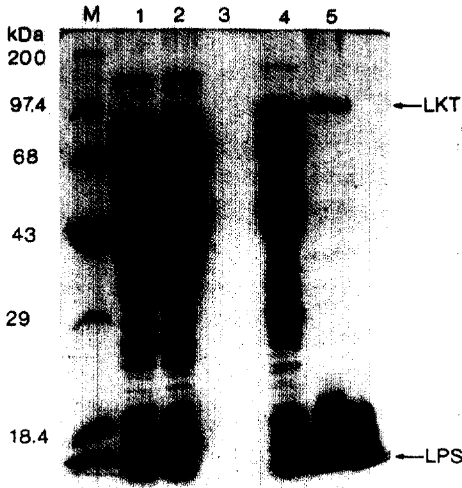

Fig 7. Silver and Coomassie Brilliant Blue staining of SDS-PAGE profiles of a various cell lysates. Lane M, molecular weight markers ; lane 1, lysate from *B subtilis* spoO12A harboring pBY2 and pBL1 ; lane 2, lysates from *B subtilis* spoO12A harboring p602/20 and pBL1 ; lane 3, buffer control ; lane 4, lysate from *E coli* DH5α harboring pTR52 ; lane 5, crude LKT from culture supernatant from *P haemolytica* A1. Silver-staining was accomplished following the procedure described in Materials and methods.

Discussion

Although bovine pneumonic pasteurellosis, commonly known as shipping fever, is a multifactorial disease involving interaction between infection by a variety of microorganisms and stress factors such as transportation, cold and malnutrition, *Pasteurella haemolytica* biotype A, serotype1(A1) has been considered as the primary agent responsible for the clinical disease and pathophysiological events characterized by acute lobar fibrinonecrotizing pneumonia[3-5]. Out of the virulence factors produced from *P haemolytica* A1, LPS and LKT have received the most attention even though all factors are likely important in the pathogenesis of the disease.

The role of LPS in the pathogenesis of the disease have been revealed clearly due to the availability of purified LPS[8-12]. However, not much is known about the contribution of LKT to the lung injury even though there are several indirect evidences suggesting the involvement of LKT in lung injury. Using immunohistochemical techniques on tissue sections of pneumonic lung,[45] we have demonstrated that LKT was associated with degenerative changes in the alveolar macrophages (AM) and PMNs. In other *in vitro* studies[19], we have shown that in addition to its cytocidal activity on bovine PMNs, the LKT also stimulated a respiratory burst and degranulation of lysosomes. Stimulation of the respiratory burst as evidenced by generation of toxic oxygen radicals was an immediate event, while cytolysis and release of proteases by degranulation occurred over longer period. It was speculated that LKT may also generate other inflammatory mediators such as inflammatory cytokines and lipid mediators in the lungs. In order to obtain direct *in vitro* and *in vivo* evidence, we need a large amount of purified LKT in the biologically active state.

Attempts to purify the LKT have been unsuccessful because it is complexed with the LPS and there is no efficient way to remove the LPS without inactivating the LKT. Traditional techniques such as ultrafiltration[46], gel permeation chromatography[47], ion-exchange chro-

matography[23], and immunoaffinity chromatography[37] have produced only partially purified LKT contaminated with LPS. Recently, we developed a new method to purify LKT using preparative SDS-PAGE, Model 491 Prep cell, in our laboratory[48]. However, we need to develop more efficient methods to prepare starting crude materials that was free of LPS. The intent of this reserach was to use recombinant DNA technology and produce large quantities of LPS free crude LKT. We used *B subtilis* as the host bacteria for this purpose. *B subtilis* is apathogenic, and has the ability to express and synthesize large amounts of the heterologous proteins[26]. Because it is a Gram-positive bacteria, it is devoid of LPS contamination. However, in the cloning and expression of a gene in *B subtilis*, one can encounter several disadvantages, namely a low transformation frequency and instability of the plasmid for direct cloning because of rare availability of its own plasmid vector[31,32]. Therefore, we used shuttle expression vectors in this experiment. Furthermore, in order to avoid the possibility of cytotoxic effect of the LKT to host bacteria, dual-plasmid repressible system was used to express *P haemolytica* LKT in *B subtilis*. In the expression of LKT in *B subtilies*, the LKT was expressed without IPTG induction even though it was in lower concentration in comparison with the expression after IPTG induction. This phenomenon was probably due to incomplete inhibition of the promoter in p602/20 by *lac*I from pBL1. The highest expression of rLKT in *B subtilis* was shown at 2h after IPTG induction. After 2h, the amount of protein expressed in *B subtilis* was decreased as revealed by Western blot analysis using MAb601 as a probe. This decrease may be due to either proteases from *B subtilis*[49,50] or instability of the transformed expression vector, pBY2, in *B subtilis* due to its carrying a big insert[26,31]. The expression of rLKT by *B subtilis* as shown by Western blot analysis, was predominantly intracellular proteins although low amount of extracellular expression was identified as rLKT(Fig 3). The low amount of secreted proteins might be due to the poorer recognition of signal peptide because LKT has C-terminal signal peptide instead of N-terminal signal peptide[14,52,52]. The other possibility may be the differences in configuration or hydrophobicity of the protein (rLKT) to pass through the cell membrane of *B subtilis*.

In order to increase the efficiency of expression of the LKT in *B subtilis*, the sucrose-inducible promoter(*Sac*B) from *gro*ESL was introduced into *lkt*CA genes of pHPY1. Also, three different strains developed for expression of foreign proteins in *B subtilis* were used. *B subtilis* Raj1105 harboring pBY3 grown overnight in the presence of 2% sucrose showed the highest expression. However, the amount of proteins expressed in *B subtilis* was low amounts, less than 1% in comparison with total proteins of *B subtilis*. This is probably due to instability of the recombinant plasmid in *B subtilis* during bacterial replication because of the large size of insert[26,31].

In order to determine the biological activity (cytotoxicity) of LKT produced from *P haemolytica*, several different methods have been developed such as the colorimetric assay using MTT dye[43], visual assay[23], colorimetric XTT dye assay[53] and neutral red uptake assay[42]. We used the visual assay and neutral red uptake assay because the colorimetric assay using MTT or XTT dye proved difficult to be adapted to quantify rLKT. We found that some substance(s) present in the cellular lysates of *B subtilis* interfered with the reduction of the MTT or XTT dye to formazan. In the visual assay(Fig 5), morphological changes of BAMs exposed to cellular lysates of *B subtilis* harboring pBY2 and pBL1 were similar to those of BAMs exposed to crude LKT produced from *P haemolytica* A1. Also, there was difference in percent cytotoxicity of BAMs exposed to cellular lysates of *B subtilis* harboring between pBY2 and pBL1 and p602/20 and pBL1(vector control) in neutral red uptake assay(Fig 6). This cytotoxicity was susceptible to neutralization by a monoclonal antibody to *P haemolytica* leukotoxin. Based on these results, the rLKT expressed in *B subtilis* was biologically active but was not present in high concentration. Furthermore, this rLKT was free of LPS as determined by silver staining of SDS-PAGE gels (Fig 7).

In summary, it was possible to produce biologically active crude rLKT by expression of *lkt* CA genes in *B subtilis* and avoid LPS contamination. However, several prob-

lems have to be addressed before we embark on the next phase of experiments. One of the problems is the instability of the recombinant plasmid harbored in *B subtilis*, especially an expression vector carrying a large sized insert. Another problem is to find a new strain of *B subtilis* for transformation that does not produce any proteases. Once these problems are resolved, we are optimistic that we can succeed in expressing LKT at large amounts in *B subtilis*.

Conclusion

This experiment was carried out with the intention to produce large quantities of *P haemolytica* A1 crude leukotoxin (LKT) free of LPS by expression of the LKT genes (*lkt*CA) in *B subtilis*. In order to achieve this goal, first, the pLKT52 was partially digested with *Sau*3A1. From the partial digests, 3-5 kb fragments were purified, ligated with pUC18 and transformed into *E coli* NM522. Transformants generated from the purified fragments carried in pUC18 were screened for LKT production by colony blot using a monoclonal antibody against LKT, MAb601, as a probe. Positive clones were characterized by restriction endonuclease analysis, sequencing and were confirmed for LKT production by Western blot assay. One clone out of nine positive clones was selected and the *lKt*CA insert was purified and subcloned into shuttle vectors. The expression of *P haemolytica* LKT in different shuttle vectors(pHPS9, p602/20, and pHPS9-*Sac*) and different *B subtilis* hosts strains(spoO12A, BR121, WB30 and Raj1105) were analyzed by Western blot. Optimal expression of *P haemolytica* LKT was accomplished with a dual plasmid system with p602/20 carrying *lkt*CA insert (pBY2) and pBL1 in *B subtilis* spoO12A by IPTG induction for 2h. Cellular lysates of *B subtilis* spoO12A harboring pBY2 and pBL1 were used to evaluate the biological activity of crude recombinant (rLKT) expressed in *B subtilis*. Results showed that crude rLKT exhibited biological activity against bovine alveolar macrophages using the visual and neutral red uptake assays. Data from silver staining followed by Coomassie Brilliant Blue staining of SDS-PAGE patterns of crude rLKT revealed that it was free of LPS. Our results also showed that the recombinant p602/20 carrying the *lkt*CA(pBY2)genes was unstable. Furthermore, the concentration of rLKT produced by *B subtilis* was low because it was destroyed by the extracellular proteases produced by the bacteria themselves.

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