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Generation of transposon insertion mutants from type A *Pasteurella multocida*

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Abstract : The transposon Tn*phoA* was used to generate avirulent mutants from a type A *Pasteurella multocida*. A suicide vector plasmid pRT733 carrying Tn*phoA*, having the kanamycin resistant gene and harbored in *Escherichia coli* K-12 strain SM10(λ*pir*), was mated with streptomycin resistant *P. multocida* P-1059 strain as recipient. This resulted in the generation of two Tn*phoA* insertion mutants (transconjugants, tc95-a and tc95-b) which were resistant both to kanamycin (Km^R) and streptomycin (Sm^R), secreted alkaline phosphatase, and were avirulent to turkeys. Southern blot hybridization using two probes derived from internal fragments of Tn*phoA*, confirmed the insertion of Tn*phoA* into 12.9kb or 13.7kb DNA fragment from the *EcoR*V digested genomic fragments of transconjugants. The two transconjugants, tc95-a and tc95-b, were distinguishable from their parent strains by differences in ribotypes, and outer membrane protein profiles. Tn*phoA* insertion in both transconjugants also resulted in constitutive expression of a 33 Kd iron regulated outer membrane protein (IROMP). The gene encoding Sm^R was also located within the same 12.9kb *EcoR*V genomic fragment from both transconjugants. Furthermore, our finding that the recipient *P. multocida* P-1059 Sm^R strain and both transconjugants were avirulent to turkeys suggest that the either 12.9kb or 13.7kb genomic DNA contains the virulence gene and speculate that the presence of Sm^R gene or Tn*phoA* insertion may be responsible for regulating and inactivating the gene(s) encoding virulence in *P. multocida*.

Key words : *Pasteurella multocida* ; Tn*phoA* ; suicide vector ; transconjugants.

Introduction

Pasteurella multocida capsular type A is the etiologic agent of avian pasteurellosis[1], which is a highly contagious disease that causes severe losses in domestic and wild fowl throughout the world. The course of the disease can range from hyperacute to chronic[1]. The hyperacute and acute

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diseases are characterized by septicemia, purulent pleuropneumonia, irreversible damage to the spleen and liver, and eventual death caused by endotoxin[1-3]. Some progress have been made in delineating the pathogenesis of *P. multocida*. These studies have shown that virulent strains which are capable of producing septicemia, are invasive, resistant to the bactericidal activity of chicken or turkey plasma and evade the phagocytic defense system[4-6]. Resistance to the bactericidal activity of plasma is due to the presence of capsular polysaccharide on the organism[5]. In addition, resistance to phagocytosis and killing by avian leukocytes are attributed to capsular polysaccharide[6] and a 50Kd antiphagocytic outer membrane protein (OMP)[7]. In addition, the lipopolysaccharide (LPS) from *P. multocida*, which has endotoxin properties, appears to be the major component responsible for the symptoms and death associated with the disease[2]. More recently, there is speculation that the iron uptake system of avian strains of *P. multocida* which enable the bacteria to scavenge iron and facilitate multiplication *in vivo*, may also contribute to virulence[8]. Despite these extensive studies on the role of various factors that contribute to the virulence of *P. multicoda*, very little is known about the genes encoding these virulence factors.

The control of pasteurellosis in domestic fowl (chickens and turkeys) remains a problem despite the availability of bacterins and live vaccines for immunization. In North America, the most widely used immunizing agent for controlling pasteurellosis in chickens and turkeys are the live vaccines prepared with the avirulent CU and M9 strains of *P. multocida*. These live vaccines induce protection against both homologous and heterologous serotypes by production of cross-protection immunogens[9]. However, two serious disadvantages encountered with the liver vaccines are that they induce protective immunity that never lasts beyond 4 weeks[10], and when used in immunocompromised domestic fowl, they cause clinical disease and death[11]. Because of these drawbacks, there is a need to develop better avirulent live vaccines that are safe, yet provide a long lasting and a broad spectrum of protection against all the somatic serotypes of *P. multocida*. Understanding the genetic basis of virulence in *P. multocida* should aid in the progressive development of a new generation of better live avirulent vaccines to adequately control this important disease.

Since the application of the antibiotic-resistant transposons was reported to facilitate the genetic analysis and molecular cloning of prokaryotic genes[12], transposon mutagenesis have been used successfully to identify the genes encoding virulence determinants in a variety of bacterial pathogens[13,14]. Weiss *et al*[15] used transposon induced mutations to identify virulence factors and protective antigens encoded by the virulence region of the *Bordetella pertussis* chromosome. The transposon Tn*phoA* (Tn5 IS 50L : : phoA), when used as a mutagen, generates mutants by insertion of Tn*phoA* into a gene encoding a secreted protein. The result is the creation of a hybrid polypeptide which is a fusion product of the polypeptide encoded by the interrupted gene and transposon-encoded alkaline phosphatase[16]. Therefore, Tn*phoA* mutagenesis can facilitate the detection of mutants that secrete proteins into the bacterial envelope and also eliminate mutants that do not secrete proteins from cytoplasmic membrane.

In order to enhance our understanding of the role of virulence factors in pathogenesis and protective immunity in avian pasteurellosis, a better understanding of the genes encoding these factors is needed. For these reasons, we decided to generate Tn*phoA* insertion mutants in an avian *P. multocida* strain and describe the characteristics of two avirulent Tn*phoA* mutants (transconjugants).

Materials and Methods

Bacteria, plasmids, and growth conditions : A streptomycin resistance (Sm^R) *P. multocida* mutant (P-1059 Sm^R) derived from the wild P-1059 virulent strain was obtained by metabolic drift mutation[30], and used as the recipient. *Escherichia coli* K-12 strain SM 10 (λ*pir*) harboring the suicide plasmid pRT 733 carrying Tn*phoA* was kindly provided by J.J. Mekalanos, Harvard University, Cambridge, Mass. Luria-Bertani (LB) agar, LB broth, or brain heart infusion (BHI) broth containing appropriate antibiotics were used as maintenance media, unless otherwise stated. The concentration of antibiotics used was as follows : ampicillin

(Ap), 50mg/ml ; kanamycin (Km), 50mg/ml ; streptomycin (Sm), 100mg/ml.

Chemicals and enzyme : All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction enzymes and other enzymes were purchased from either Gibco, BRL., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, IN. Non-radioactive labeling kit was purchased from Boehringer Mannheim.

Generation of *P. multocida* Tn*phoA* insertion mutants : The donor strain *E. coli* K-12 SM10 containing the suicide vector pRT 733 carrying Tn*phoA* was mated P-1059 Sm^R for 4 to 6h at 37℃ on LB agar plates using a standard procedure[14]. After mating, a loopful of growth was restreaked on LB agar containing Km (100mg/ml) and Sm (100mg/ml), to select transconjugants harboring Tn*phoA*. Initial selection of transconjugants was done on the basis of acquisition of kanamycin resistance (Km^R) which should arise by transposition of Tn*phoA* from pRT 733 to the recipient genome. The Sm^R and Km^R transconjugants were then restreaked onto LB agar containing Km (50mg/ml), Sm (100mg/ml), and the chromogenic substrate for alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine (XP-BCIP, 25mg/ml) (Sigma), followed by incubating at 37℃ for 24 to 48h. The plates were stored at 4℃ up to 1 week to allow appearance of blue colored colonies. Individual blue colonies (transconjugants) were then confirmed for the production of alkaline phosphatase using a previously described procedure[32], Units of alkaline phosphatase activity was calculated using the formula described by Brickman and Beckwith[33]. Transconjugants containing transpositional insertions and producing fusion proteins that showed alkaline phosphatase activity above were identified and stored at -70C for further study.

DNA manipulation techniques : Isolation and purification of plasmid DNA, chromosomal DNA, restriction enzyme treatment, and agarose gel electrophoresis were accomplished by standard techniques described by Sambrook *et al.*(1989)[32].

Detection of transposon in transconjugants by Southern blot hybridization : A 1.3kb fragment from Tn*phoA* was obtained by digesting pRT 733 with *EcoRI-XhoI* and purifying the fragment by alkaline lysis method[32]. This 1.3kb fragment was subcloned in pcDNA II (Invitro-gene Corp., San Diegeo, CA). A 7kb DNA fragment obtained from pRT 733 digested with *DraI-HpaI* was also used as a probe for the same purpose. DNA probes were labeled with digoxygenin and nonradioactive DNA hybridi-zation was performed using the method described by Holtke *et al*[34]. Results were visualized by chemilumigram using X-Omat AR film (Eastman Kodak Co., Rochester, Y.Y.) exposed at room temperature.

Characterization of transconjugants :

Outer membrane protein (OMP) profiles : Overnight cultures of the same 5 strains grown in tryptic soy broth were centrifuged, washed twice in PBS and suspended in HEPES buffer (10mM, pH 7.4). Outer membrane proteins (OMPs) were extracted by the method described by Choi *et al*[35], and subjected to SDS-PAGE analysis[36]. Protein concentration was measured by Lowry method[37].

Western blot analysis : OMPs from 4 different strains were separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane as described by Towbin *et al*[38]. Turkey convalescent-phase serum or anti-IROMP serum was used as the primary antibody. The secondary antibody was affinity-purified either goat anti-turkey IgG (H+L) or goat anti-chicken IgG (H+L) labeled with horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Visualization of bands were performed as described by Choi *et al*[35].

Ribotyping : *E. coli* 16S rRNA (Sigma) was suspended in sterile distilled water to a concentration of 0.25mg/ml. The rRNA was reverse transcribed into cDNA with reverse transcriptase. The cDNA was then labeled with digoxigenin according to the manufacturer's directions (Boehringer Mannheim). Hybridization was performed on nylon membranes[32] and visualization was done as described earlier.

Determination of virulence for turkeys : A turkey mortality test was performed to assess virulence of various *P. multocida* strains. One day old, specific pathogen-free (SPF) turkey poults were obtained and raised in isolation units 7 weeks of age. Seven turkeys belonging to each of the 4 groups were injected intravenously with 0.5ml of a late logarithmic phase growth bacterial suspension containing 5×

10^4CFU of P-1059 wild strain, recipient strain P-1059 Sm^R, or the two transconjugants, tc95-a and tc95-b. Seven turkeys used as controls were injected with 0.5ml of normal saline intravenously. All turkeys were observed for 3 weeks and mortality was recorded. All dead turkeys were subjected to postmortem and bacteriological examination, to confirm the presence of fowl cholera disease.

Antisera : Turkey convalescent-phase serum was obtained from turkeys, one month after recovery from experimental avian pasteurellosis. Monospecific antibodies directed against the iron-regulated outer membrane proteins (IROMPs) of *P. multocida* was produced by immunizing SPF chickens with purified IROMPs from SDS-PAGE. *Pasteurella multocida* P-1059 wild strain was grown in an iron-restricted medium and an IROMP-enriched extract was prepared as described by Choi *et al*[27]. The IROMPs were identified after SDS-PAGE and the 76Kd, 84Kd, and 94Kd bands were cut and frozen at -70C. The frozen bands, containing the IROMPs, were ground up in PBS, and the resulting slurry was emulsified in an equal volume of Freund's incomplete adjuvant. Five, 6-week-old SPF chickens were immunized with 500mg of IROMPs mixed with adjuvant, intramuscularly. The animals were injected two times at 2 weeks interval. Serum from all chickens was obtained prior to immunization and 4 weeks after the last immunization. Pre- and post-immunization sera were pooled separately, filter-sterilized and stored at -80C. The specificity of the antiserum to IROMPs was determined by Western blot analysis[38].

Synthesis of oligonucleotides primers : Two, 20bp oligonucleotide primers which flank the published sequence at the 5' and 3' ends of the streptomycin resistance (Sm^R) gene from *P. haemolytica*[39] were synthesized on a model 391 DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). These primers were used for amplification of the Sm^R gene by polymerase chain reaction (PCR).

PCR amplification : Five hundred micro gram of DNA was amplified through 30 cycles of PCR. The primer pairs were used to amplify the Sm^R gene in P-1059 Sm^R and the two transconjugants. The reagents for PCR included, 500μg of sample DNA, 200 pmol of each primers, 225mm of each dATP, dCTP, dGTP, and dTTP, 25mM $MgCl_2$ and 5 units of Taq polymerase (Promega Co., Madison, WI). A 50μl of mineral oil (USB, Cleveland, OH) was overlayed on the top of the reaction mixture to protect against evaporation. Each cycle started with 1 min denaturation at 95C, followed by cooling, and incubating at 55C for 1 min to allow primer annealing. Each cycle ended with a chain elongation step of 2 min at 72C.

Detection of streptomycin resistance gene : Two methods were used to detect the presence of Sm^R gene in various *P. multocida* strains was detected either by PCR amplification of Sm^R gene from pYFC5 plasmid (provided by Y.F. Chang, Cornell University, Ithaca, N.Y)(data not shown). Second, the plasmid pYFC5 carrying the Sm^R gene of *P. haemolytica*[39] was purified and was labeled with digoxigenin. *Eco*RV digests of genomic DNA from various strains were Southern blotted, hybridized with the probe and the Sm^R gene was identified. The pYFC5 plasmid was used as positive controls for the presence of Sm^R gene, while the PCR products from the wild P-1059 strain served as negative controls.

Result

Isolation of *P. multocida* Tn*phoA* transconjugants : Our aim was to obtain avirulent Tn*phoA* insertion mutants from *P. multocida* P-1059 Sm^R using *E. coli* K-12 SM10 (*λpir*) containing a suicide vector *pRT* 733 as the Tn*phoA* donor. *Pasteurella multocida* P-1059 Sm^R was chosen for use as recipient strain, because it was derived from a highly virulent P-1059 wild strain that had no demonstrable plasmid and can be used streptomycin resistance as a selection marker for recipient. Approximately, 4,500 both kanamycin resistant (Km^R) and streptomycin (Sm^R) transconjugants were obtained after mating strain P-1059 Sm^R with *E. coli* donor strain. These Km^R and Sm^R transconjugants were further screened on LB agar containing the antibiotics and 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine (XP-BCIP). Transconjugants containing transpositional insertions and producing fusion proteins that showed alkaline phosphatase activity (PhoA+) detected by blue colored colonies were identified. Forty two clones which were Km^R, Sm^R

and PhoA+, were selected. Two out of 42 individual blue colored colonies, tc95-a and tc95-b, were found to be avirulent as assessed by a turkey mortality test.

Confirmation of alkaline phosphatase activity : If the Tn*phoA* was inserted in a random manner into the genome of P-1059 Sm^R recipient strain, it should generate transconjugants that can secrete fusion proteins with alkaline phosphatase activity. Thus, the secretion of alkaline phosphatase was used as an indicator of insertion of Tn*phoA* fragment into genomic DNA of the two transconjugants. Since the synthesis of the enzyme alkaline phosphatase by *E. coli* was regulated by end-product repression, the addition of phosphate to the media would inhibit expression of the structural gene (PhoA). Induction of alkaline phosphatase activity was measured in wild P-1059 strain, recipient P-1059 Sm^R, and the two transconjugants grown in LB medium containing high to low concentrations of phosphate. A Km^R and Sm^R clones which did not display the blue color on the plate containing XP-BCIP, were used as a negative control for alkaline phosphatase. The donor strain *E. coli* (pRT 733) was used as positive control. The highest alkaline phosphatase activity was detected in the *E. coli* donor strain. Alkaline phosphatase activity was higher in the transconjugant tc95-b than in tc95-a (Table 1). Insignificant amount of alkaline phosphatase was secreted by strains P-1059 and P-1059 Sm^R compared to the negative control.

Table 1. Measurement of alkaline phosphatase activity (AP)

Strains	AP activity[a]
tc95-ng1[b]	128
tc95-ng2[b]	67
P-1059	183
P-1059 Sm^R	168
tc95-a	436
tc95-b	608
E. coli (pRT733)[c]	763

a : $1{,}000 \times \frac{OD420 - 1.75 \times OD550}{OD600} \times$ dilution factor

b : Negative controls.
c : Positive control.

These results provided indirect evidence that Tn*phoA* was integrated into the genomic DNA of the two transconjugants, tc95-a and tc95-b, and the fusion protein (s) was secreted into the extracytoplasmic membrane.

Southern blot and analysis of *P. multocida* Tn*phoA* transconjugants : To confirm the integration of Tn*phoA* DNA fragment into the two transconjugants and to identify the gene (s) responsible for virulence, Southern blot hybridization was performed using a EcoRI-XhoI digested 1.3kb or DraI-HpaI digested 7kb fragments derived from pRT 733, as probes. Chromosomal DNA from *P. multocida* strains P-1059 and P-1059 Sm^R, tc95-a and tc95-b, and *E. coli* donor strain, were digested with *Eco*RV and hybridized with the probe. The 1.3kb probe hybridized to a 12.9kb fragment from tc95-a and to 13.7kb and 12.9kb DNA fragments from tc95-b (Fig 1). The same 13.7kb DNA fragment was also detected from *E. coli* donor strain. In contrast, no hybridization was observed with DNA from the wild P-1059 or P-1059 Sm^R strains. Southern blot profiles using the same chromosomal DNA digests probed with the 7kb fragment were identical to that hybridized with the 1.3kb probe (data not shown). These results suggest that Tn*phoA* was integrated into the genomic DNA of tc95-a and tc95-b and since both were avirulent as assessed by the turkey mortality test, the 12.9kb

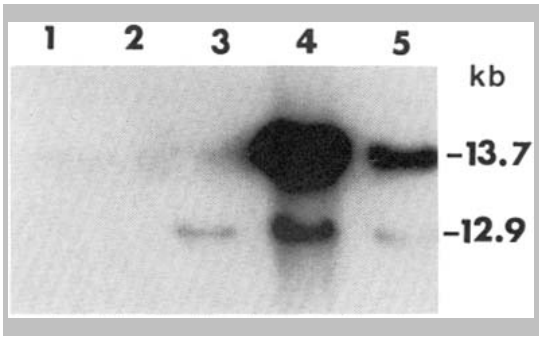

Fig 1. Southern blot hybridization for detection of Tn*phoA* gene. Chromosomal DNA from P-1059, P-1059 Sm^R, tc95-a, tc95-b, and *E. coli* donor strains was digested with *Eco*RV and hybridized with 1.3kb probe. The 1.3kb probe hybridized to a 12.9kb fragment from tc95-a (lane 3) and to 13.7kb and 12.9kb DNA fragments from tc95-b (lane 4). The same 13.7 kb DNA fragment was also detected from *E. coli* donor strain (lane 5). In contrast, no hybridization was observed with DNA from the wild P-1059 or P-1059 Sm^R strains (lanes 1 and 2, respectively).

or the 13.7kb DNA fragments from tc95-a and tc95-b may contain the gene (s) encoding virulence determinants of *P. multocida*.

SDS-PAGE and Western blot analysis : SDS-PAGE and Western blot performed characterization of membrane proteins. Outer membrane proteins (OMPs) from P-1059, P-1059 Sm^R, and the two transconjugants were subjected to SDS-PAGE, and Western blot analysis. SDS-PAGE profiles of OMPs from P-1059 were similar to that of OMPs extracted from P-1059 Sm^R except 94.0Kd protein expressed by P-1059. SDS-PAGE profiles of OMPs from the two transconjugants were similar, but not identical, and different that from the P-1059 and P-1059 Sm^R strains (Fig 2a). While tc 95-a and tc95-b expressed two major OMPs with molecular masses 36.3Kd and 33Kd, strains P-1059 and P-1059 Sm^R expressed OMPs of 37.2Kd and 34.4Kd. This 33Kd OMP expressed by the two transconjugants showed intense reactivity with antibodies present in anti-IROMP serum (Fig 2b). By contrast, the 37.2Kd OMP expressed by P-1059 and P-1059 Sm^R showed no reactivity with anti-IROMP serum (Fig 2b).

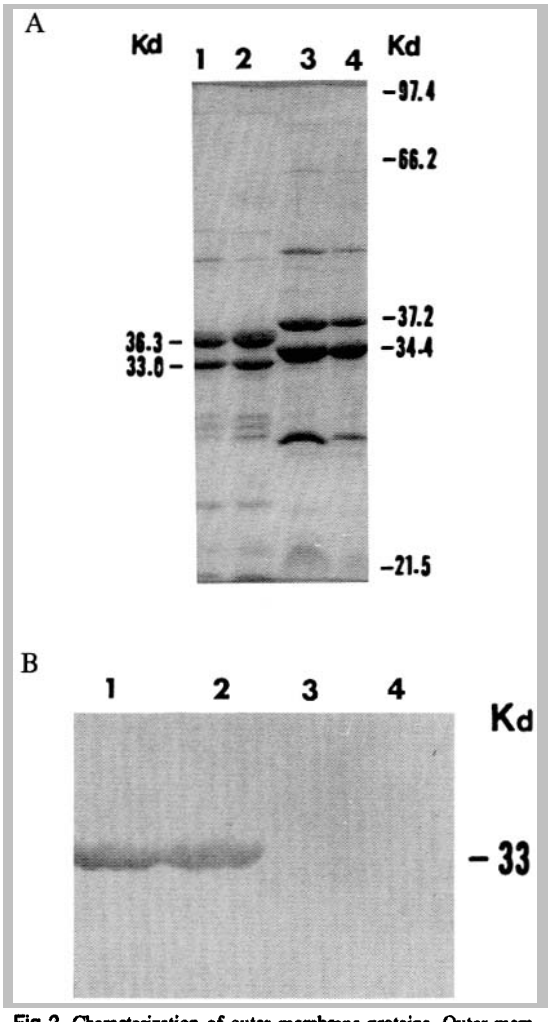

Fig 2. Characterization of outer membrane proteins. Outer membrane proteins (OMPs) extracted from tc95-b (lane 1), tc95-a (lane 2), P-1059 Sm^R (lane 3), and P-1059 (lane 4) were subjected to SDS-PAGE (a). The profile of Western blotting (b) carried out using anti-iron regulated OMPs serum was shown in tc95-b (lane 1), tc95-a (lane 2), P-1059 Sm^R (lane 3), and P-1059 (lane 4).

Ribotyping : Genomic fingerprinting by ribotyping was used to determine whether restriction-fragment-length-polymorphism (RFLP) existed in the genomic DNA of the strains. Genomic DNA from the two transconjugants, P-1059, P-1059 Sm^R, and *E. coli* donor strain were digested with *EcoR*V or *EcoR*1, blotted onto nylon membrane and hybridized using a cDNA probe to the 16S rRNA from *E. coli*. Ribotype of *EcoR*V restriction fragments from P-1059 and P-1059 Sm^R strains were identical (Fig 3a). Although, the *EcoR*V restriction fragments from tc95-b and the *E. coli* donor strain had the same ribotype profile, it was distinctly different from the profile of P-1059 and P-1059 Sm^R. In addition, *EcoR*1 restriction fragments from tc95-b was different from *E. coli* donor stain (Fig 3b). In contrast, the ribotype profile of tc95-a digested with *EcoR*V revealed a high degree of polymorphism, in that it had a mixture of hybridization bands from the donor *E. coli* and recipient P-1059 Sm^R(Fig 3a). Ribotype of *EcoR*1 restriction fragment from tc95-a was identical to that of recipient strains, P-1059 or P-1059 Sm^R (Fig 3b).

Determination of virulence : To assess virulence of the two transconjugants, P-1059, and P-1059 Sm^R strains, a mortality test using turkeys was performed. As shown in Table 2, all turkeys inoculated with P-1059 strain were dead in 18h. These turkeys showed clinical symptoms of fowl chloera such as anorexia, diarrhea, fever, and endotoxemia. All dead turkeys subjected to necropsy showed gross pathological lesions of fowl cholera. In contrast, there were no clinical symptoms or mortality in turkeys inoculated with the two

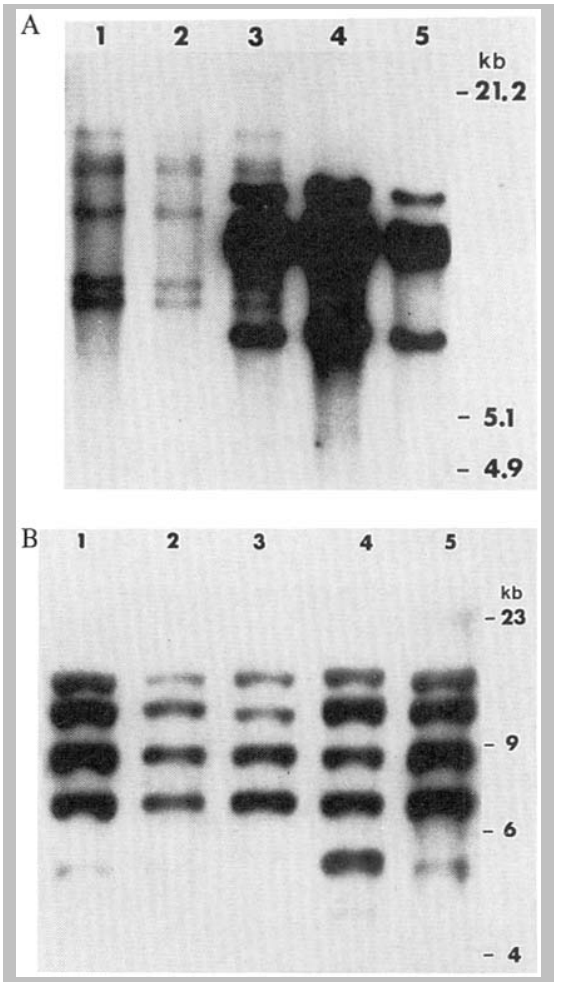

Fig 3. Ribotyping. Chromosomal DNA from P-1059 (lane 1), P-1059 Sm^R (lane 2), tc95-a (lane 3), tc95-b (lane 4), and *E. coli* donor strain (lane 5) were digested with either *EcoRV* (a) or *EcoRI* (b), and hybridized with cDNA probe to the 16S rRNA from *E. coli*.

Table 2. *In vivo* assessment of virulence

Strains	Mortality (%)
P-1059	100
P-1059 Sm^R	5
tc95-a	0
tc95-b	0
Control	100

[a]: (1-Number of survived / Number of inoculated) × 100.

transconjugants, tc95-a and tc95-b. To our surprise, turkeys challenged with *P. multocida* P-1059 Sm^R recipient strain also showed no clinical symptoms or mortality. These turkeys were euthanized three weeks after challenge, necropsied, and isolation of *P. multocida* was attempted from the heart, lungs, liver, and spleen tissues. No lesions suggestive of fowl cholera disease were observed and *P. multocida* were not isolated from any of the tissues.

Detection of streptomycin resistance gene in the transconjugants : Since the recipient P-1059 Sm^R strain and the two transconjugants were avirulent to turkeys, it was necessary to determine the location of the Sm^R gene in relation to the Tn*phoA* insertion in the chromosomal DNA of the two transconjugants. Two experiments were performed to obtain some answers to this question. First, PCR amplification of the genomic DNA from P-1059 Sm^R, two transconjugants, and plasmid DNA from pYFC5 produced a 720bp band detected by gel electrophoresis. By contrast, this band was ab-

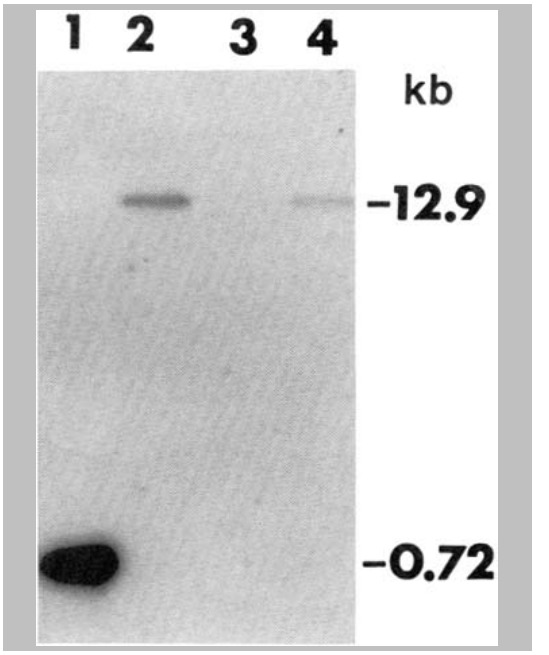

Fig 4. Detection of streptomycin resistant gene by Southern blot hybridization. Southern blot analysis showed hybridization of genomic DNA from tc95-a (lane 2) and tc95-b (lane 4) with the 720bp *strA* gene probe. No hybridization was observed with the genomic DNA from the P-1059 strain (lane 3). The pYFC5 plasmid (lane 1) as a positive control showed 720bp DNA fragment by Southern blot analysis.

sent in the PCR products derived from amplification of genomic DNA from the P-1059 and *E. coli* donor strain (data not shown). Second, Southern blot analysis revealed that a 12.9kb *EcoR*V restriction fragment from genomic DNA frm the two transconjugants, tc95-a (lane 2) and tc95-b (lane 4), hybridized with the 720bp *strA* gene of PCR production from pYFC5 plasmid DNA of *P. haemolytica*. However, no hybridization was observed with the genomic DNA from the P-1059 strain (lane 3). The pYFC5 plasmid (lane 1) as positive control showed 720bp DNA fragment by Southern blot analysis (Fig 4).

Discussion

In this study, transposon mutagenesis using Tn*phoA* was adopted to generate fusion strains defective in virulence gene(s) of type A *P. multocida*. This system demonstrated the identification of fusion to random genes expressing secreted proteins[17]. Furthermore, alkaline phosphatase coding sequence was fused downstream of target gene sequences encoding secretion signal and the insertion of Tn*phoA* appeared to occurr non-specifically[18]. In addition, the suicide vector was able to enhance defined mutations in genes that encode secreted proteins in the absence of any selection or previous information[19]. *E. coli* donor strain harboring the suicide vector pRT 733 carrying Tn*phoA* was mated with *P. multocida* P-1059 Sm^R as recipient and mutants were isolated having both antibiotic resistance and alkaline phosphatase activity. The suicide vector, pRT 733, cannot replicate in the recipient strain because of the lack of p protein encoded by λ*pir* transducing phage[20]. Two Tn*phoA* insertion mutants (transconjugants), tc95-a and tc95-b, were generated which were Km^R, Sm^R, and PhoA+ and avirulent to turkeys.

Our findings that the secretion of alkaline phosphatase was higher in two transconjugants, tc95-a and tc95-b than in P-1059 and P-1059 Sm^R strains (Table 1), provided indirect evidence that Tn*phoA* was transposed into the genomic DNA of *P. multocida* P-1059 Sm^R and a fusion product was secreted. Direct evidence that Tn*phoA* was indeed integrated into the genomic DNA of the two transconjugants, was provided by Southern blot hybridization which showed a 12.9 kb *EcoR*V fragment from tc95-a hybridized with two Tn*phoA* derived probes, and 13.7kb and 12.9kb fragments from tc95-b hybridized with the same probes. Furthermore, a 13.7kb *EcoR*V fragments from the genomic DNA of *E. coli* donor also hybridized with the same probes.

Additional data presented here demonstrated that the transconjugants could be differentiated from the two parent strains and the P-1059 strains of *P. multocida*. The OMP profiles of tc95-a and tc95-b were similar to the donor strain except 45Kd and 53Kd proteins. The 36.6Kd and 33Kd were major band among two transconjugants and the donor strain. By Contrast, the OMPs of strains P-1059 and P-1059 Sm^R were similar but different from the two transconjugants in that they expressed major OMPs in the 37.2Kd and 34.4 Kd regions. Convalescent-phase serum contained antibodies that reacted intensely with the 33Kd OMP expressed by the transconjugants and with the 37.2Kd OMP expressed by P-1059 and P-1059 Sm^R strains. Genomic fingerprinting by ribotyping using *EcoR*V or *EcoR*1 restriction fragment revealed that two transconjugants were not identical to both parent strains. In addition, the demonstration of restriction fragment length polymorphism in the genomic DNA of tc 95-a and tc95-b using ribotyping and restriction enzyme finerprinting (data not shown) suggested that they were not genetically identical. Finally, no ill effects were observed in turkeys following intravenous challenge with tc95-a or tc95-b, compared with challenge with the wild *P. multocida* P-1059 strain. The latter killed all turkeys overnight. Surprisingly, turkeys challenged with the *P. multocida* P-1059 Sm^R recipient strain also showed no ill effects. Results from additional experiments showed that the Sm^R gene and Tn*phoA* were localized within the 12.9kb or 13.7kb *EcoR*V fragment of the genomic DNA from transconjugant. Since transconjugants and P-1059 Sm^R were avirulent to turkeys, we speculate that this 12.9kb or 13.7kb fragment may also contain the virulence gene(s) of *P. multocida*. It is quite possible that the Sm^R gene by some unknown mechanism yet to be determined in this study may be inactivating the virulence gene(s). The Sm^R gene and Tn*phoA* insertion are located in "hot spot" areas of the chromosome and may cause

inactivation of the virulence gene(s).

The genetic control of virulence in influenced by random and non-random factors. Phenotype change, which is referred to as "phase" or "antigenic" variation, is caused randomly and is detected by alterations of surface structures such as flagella, pili, OMP, or capsule[21,22]. In non-random control, pathogenic bacteria sense signals in the environment and respond by expressing necessary gene products for survival in the host[23]. The expression of bacterial virulence determinants may also be controlled by other factors, and a wide spectrum of compounds and growth conditions have been implicated in the regulation of virulence properties including iron[24], divalent cations[25], and temperature[26]. Survival in the multigeneric host defense system requires that a microbe not only evade host defense systems but also overcome the stringent competition for nutrients from its neighboring bacterial competitors and from the nutritional alteration in the host, especially available iron. We[27] and Others[28,29] have reported that the absence of iron induced the expression of novel immunogenic antigen(s) and was also an important regulatory factor in the physiology and pathogenesis of *P. multocida*. Furthermore, absence of iron also increased the virulence of *P. multocida* in mice[29]. In this study, we found that Tn*phoA* insertion in the transconjugants also resulted in the expression of a 33Kd OMP when transconjugants were grown in iron-deficient or iron-replete medium. Previous studies from our laboratory showed that the wild P-1059 strain of *P. multocida* grown in an iron-deficient medium expressed IROMPs with molecular masses 76Kd, 84Kd, and 94Kd[27]. The fact that this 33Kd OMP reacted with monospecific antibodies directed against the IROMPs of *P. multocida* in Western blots, suggest that it is an IROMP although smaller in size. It is conceivable that the reduced size of IROMP constitutively expressed by both transconjugants may be caused by mutation resulting from Tn*phoA* insertion in the structural gene causing the synthesis of a truncated IROMP which contained epitopes that bind to antibodies. Further studies are underway to clarify the molecular basis of the constitutive expression of a 33Kd IROMP by the two transconjugants.

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