Introduction

*Pasteurella multocida* causes fowl cholera in turkeys and chickens, and many avian species, and hemorrhagic septicemia in cattle and buffalos, and atrophic rhinitis in swine. The capsule of *P. multocida* type A is most often associated with avian cholera, and functions as a virulence factor, and it is composed largely of hyaluronic acid [1,2]. Strains belonging to capsular types B, D and F of *P. multocida* have also been isolated from diseased birds, but with low incidence as compared to capsular type A [3,4].

The capsulated strains were more virulent than the noncapsulated strains of the virulent *P. multocida*, and the noncapsulated strains of virulent isolates are able to infect, but not to cause mortality [5,6]. A spontaneous noncapsulated mutant P-1059B obtained from 35 serial passages of *P. multocida* strain P-1059, demonstrated that the loss of ability to produce capsular materials resulted in a marked loss of virulence [7]. A capsulated strain P-1059 was shown to resist the action of complement compared to a noncapsulated strain P-1059-1A [8]. The capsular hyaluronic acid also mediated adhesion of *P. multocida* type A strains to turkey air sac macrophages [9]. The capsulated strain of *P. multocida* treated with hyaluronidase became complement-sensitive and were more readily phagocytosed in comparison with untreated capsulated strain [10]. These studies have suggested that the capsular hyaluronic acid is a key virulence factor of *P. multocida* type A strains. However, because these strains were not genetically defined, it is not possible to ascribe definitively their phenotypes to the lack of capsule. Thus the entire capsule biosynthetic locus has been cloned and sequenced from a serotype A:1 strain X-73 of *P. multocida* [11], and sequence analysis showed that the locus containing three functional regions. Subsequently, constructed a defined acapsular mutant of the strain X-73 by disrupting the *hexA* gene through the insertion of a tetracycline resistance cassette, demonstrated that the capsule of the organism is an essential virulence factor in both mice and chickens [12]. In this study, we constructed an acapsular mutant of *P. multocida* P-1059 by homologous recombination, and pathogenicity in chickens and protective ability of the mutant strain were evaluated.
For the PCR amplification, two specific primers P-1 (5’-ATGATC- GAAACAAAATAC-3’) and P-2 (5’-CCCTATTCTTATTTGACGATGACC-3’) were designed according to the published hexB gene and its flanking sequence of P. multocida P-1059 [10] (X. Z. Wang and H. Y. Liao). The expected size of the resulting amplicon was 991 bp in length. Genomic DNA of P. multocida P-1059 was isolated using the bacterial genomic DNA extraction kit (TaKaRa, China) according to the manufacturer’s instructions. The hexB gene and its flanking sequence was amplified from the genomic DNA of P. multocida P-1059 by PCR using the primers P-1 and P-2. The PCR product was electrophoresed on a 1.0% agarose gel and purified using an agarose gel DNA fragment recovery kit (TaKaRa, China) according to the manufacturer’s instructions. The PCR product was cloned into a pMD18-T vector to generate pMD18-hexB vector. The construction was transformed into chemically competent E. coli DH5α. The recombinants were selected onto LB plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The recombinant plasmid DNA was isolated using a plasmid purification kit (TaKaRa), and the sequence of this insert was determined.

Cloning and sequencing of hexB gene and its flanking sequence

For the PCR amplification, two specific primers P-1 (5’-ATGATC- GAAACAAAATAC-3’) and P-2 (5’-CCCTATTCTTATTTGACGATGACC-3’) were designed according to the published hexB gene and its flanking sequence of P. multocida P-1059 [10] (X. Z. Wang and H. Y. Liao). The expected size of the resulting amplicon was 991 bp in length. Genomic DNA of P. multocida P-1059 was isolated using the bacterial genomic DNA extraction kit (TaKaRa, China) according to the manufacturer’s instructions. The hexB gene and its flanking sequence was amplified from the genomic DNA of P. multocida P-1059 by PCR using the primers P-1 and P-2. The PCR product was electrophoresed on a 1.0% agarose gel and purified using an agarose gel DNA fragment recovery kit (TaKaRa, China) according to the manufacturer’s instructions. The PCR product was cloned into a pMD18-T vector to generate pMD18-hexB vector. The construction was transformed into chemically competent E. coli DH5α. The recombinants were selected onto LB plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The recombinant plasmid DNA was isolated using a plasmid purification kit (TaKaRa), and the sequence of this insert was determined.

Construction of targeting vector pWSK29∆hexB

The PCR primers were designed according to the sequenced hexB gene and its flanking sequence of P. multocida P-1059 (GenBank accession number X679409) by using the primer premier 5.0 software program. The nucleotide sequences of these primers are described in table 2. The tetracycline resistance (Tetr) gene was amplified from pBR322 plasmid by PCR using primers Tet-F and Tet-R (Table 2), and digested with Smal I and Xho I and inserted into the same restriction sites of the plasmid pWSK29 to generate pWSK29-Tet’. DNA fragments of 991 bp (with PCR primers P991F and P991R) and 976 bp (with PCR primers P976F and P976R) upstream and downstream of the hexB gene were amplified by PCR and digested with Xba I/Sma I and Xho I/Kpn I, respectively. The two DNA fragments were inserted into the multiple cloning sites of the plasmid pWSK29-Tet’ to generate the targeting vector pWSK29∆hexB.

RT-PCR analysis of hexB deletion mutant

The entire hexB gene was amplified from genomic DNA of P. multocida P-1059 by PCR using primers HexB-F and HexB-R (Table 2). The PCR product was digested with Sac I/Xba I and cloned into the same restriction sites of the pMD18-T vector using E. coli DH5α. The sequence of this insert was determined confirmed mutant locus.

Complementation of hexB deletion mutant

The entire hexB gene was amplified from genomic DNA of P. multocida P-1059 by PCR using primers HexB-F and HexB-R (Table 2). The PCR product was digested with Sac I/Xba I and cloned into the same restriction sites of the pMD18-T vector using E. coli DH5α. The sequence of this insert was determined confirmed mutant locus.

Observation of capsular structure of P. multocida strains

The P. multocida strains were grown to an optical density of 0.5 at 600 nm, and the total RNA was isolated using the RNAprep pure Cell/Bacteria kit (Tiangen, China) according to the manufacturer’s instructions. The RNA was treated with DNase to eliminate contaminating DNA, and the cDNA was synthesized using the random octamers provided in the Quantscript first-strand synthesis kit for RT-PCR analysis of hexB deletion mutant by electroporation to generate complemented strain P-1059C.

Cloning and sequencing of hexB gene and its flanking sequence

For the PCR amplification, two specific primers P-1 (5’-ATGATC- GAAACAAAATAC-3’) and P-2 (5’-CCCTATTCTTATTTGACGATGACC-3’) were designed according to the published hexB gene and its flanking sequence of P. multocida P-1059 [10] (X. Z. Wang and H. Y. Liao). The expected size of the resulting amplicon was 991 bp in length. Genomic DNA of P. multocida P-1059 was isolated using the bacterial genomic DNA extraction kit (TaKaRa, China) according to the manufacturer’s instructions. The hexB gene and its flanking sequence was amplified from the genomic DNA of P. multocida P-1059 by PCR using the primers P-1 and P-2. The PCR product was electrophoresed on a 1.0% agarose gel and purified using an agarose gel DNA fragment recovery kit (TaKaRa, China) according to the manufacturer’s instructions. The PCR product was cloned into a pMD18-T vector to generate pMD18-hexB vector. The construction was transformed into chemically competent E. coli DH5α. The recombinants were selected onto LB plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The recombinant plasmid DNA was isolated using a plasmid purification kit (TaKaRa), and the sequence of this insert was determined.

Construction of targeting vector pWSK29∆hexB

The PCR primers were designed according to the sequenced hexB gene and its flanking sequence of P. multocida P-1059 (GenBank accession number X679409) by using the primer premier 5.0 software program. The nucleotide sequences of these primers are described in table 2. The tetracycline resistance (Tetr) gene was amplified from pBR322 plasmid by PCR using primers Tet-F and Tet-R (Table 2), and digested with Smal I and Xho I and inserted into the same restriction sites of the plasmid pWSK29 to generate pWSK29-Tet’. DNA fragments of 991 bp (with PCR primers P991F and P991R) and 976 bp (with PCR primers P976F and P976R) upstream and downstream of the hexB gene were amplified by PCR and digested with Xba I/Sma I and Xho I/Kpn I, respectively. The two DNA fragments were inserted into the multiple cloning sites of the plasmid pWSK29-Tet’ to generate the targeting vector pWSK29∆hexB.

RT-PCR analysis of hexB deletion mutant

The P. multocida strains were grown to an optical density of 0.5 at 600 nm, and the total RNA was isolated using the RNAprep pure Cell/Bacteria kit (Tiangen, China) according to the manufacturer’s instructions. The RNA was treated with DNase to eliminate contaminating DNA, and the cDNA was synthesized using the random octamers provided in the Quantscript first-strand synthesis kit for RT-PCR analysis of hexB deletion mutant by electroporation to generate complemented strain P-1059C.

Observation of capsular structure of P. multocida strains

The P. multocida strains were grown to a optical density of 0.5 at 600 nm, and the total RNA was isolated using the RNAprep pure Cell/Bacteria kit (Tiangen, China) according to the manufacturer’s instructions. The RNA was treated with DNase to eliminate contaminating DNA, and the cDNA was synthesized using the random octamers provided in the Quantscript first-strand synthesis kit for RT-PCR analysis of hexB deletion mutant by electroporation to generate complemented strain P-1059C.

RT-PCR analysis of hexB deletion mutant

The P. multocida strains were grown to an optical density of 0.5 at 600 nm, and the total RNA was isolated using the RNAprep pure Cell/Bacteria kit (Tiangen, China) according to the manufacturer’s instructions. The RNA was treated with DNase to eliminate contaminating DNA, and the cDNA was synthesized using the random octamers provided in the Quantscript first-strand synthesis kit for RT-PCR analysis of hexB deletion mutant by electroporation to generate complemented strain P-1059C.
30 min. The reaction was stopped by 10-fold dilution with cacodylate buffer, and the bacteria were washed three times in cacodylate buffer by centrifugation. The bacteria were then immobilized in 2% Noble agar, washed three times in cacodylate buffer, and postfixed with 2% osmium tetroxide for 1 h. The specimens were dehydrated in graded ethanol, and embedded in an epoxy resin mixture. Thin sections of the embedded specimen were stained with uranyl acetate and lead citrate, and then observed by electron microscopy at an acceleration voltage of 75 kW at calibrated magnification.

**Determination of hyaluronic acid capsule production**

Crude capsular polysaccharides were prepared according to the previously described method [15] with slight modification. Overnight cultures of *P. multocida* strains P-1059, ∆*hexB* and its complemented strain P-1059C grown in TB medium were diluted in 20 ml of fresh TB medium to an optical density of 0.1 at 600 nm and incubated at 37°C for 15 min. Cells were harvested from 5 ml of bacterial culture by centrifugation at 7,600 x g for 15 min, washed once with sterile phosphate-buffered saline (PBS; pH 7.4), resuspended in 1 ml of PBS and incubated at 42°C for 1 h to extract the capsular polysaccharides. Viable counts were determined before and after the incubation at 42°C, after which the cells were harvested by centrifugation at 7,600×g for 15 min and supernatant containing the capsular polysaccharide was transferred to a new tube. The hyaluronic acid content in the capsular extract was determined according to the previously described method [16].

**Virulence of *P. multocida* strains for chickens**

A total of 75 specific-pathogen-free chickens, Line 22 white Leghorn chickens (Verial Vital Laboratory Animal Technology Co. Ltd., China), approximately 56-day-old were used for testing the virulence of *P. multocida* strains P-1059, ∆*hexB* and P-1059C. These strains were grown in TB medium to an optical density of 0.5 at 600 nm, and the cultures were diluted into TB medium to obtain cultures of the desired concentrations. Exact bacterial numbers in the dilutions were determined by colony plate counts of serial dilutions. Each strain was inoculated in five groups of five birds each. The chickens were intravenously inoculated with 0.3 ml of serial dilution bacterial cultures. Five birds served as untreated control. The chickens were kept in plastic isolators and observed for clinical signs for one week after inoculation.

**Serum sensitivity assays**

The sensitivity of *P. multocida* strains and *E. coli* DH5α to chicken serum was determined according to the previously described method [17] with slight modification. Briefly, whole blood was obtained from a SPF chicken, and the serum was separated by centrifugation at 800×g for 15 min. *P. multocida* strains grown on DSA plates or *E. coli* DH5α grown on LB plates at 37°C for 18 h were suspended in 0.1 M phosphate-buffered saline (PBS, pH 7.4), and the bacterial suspensions were adjusted to the concentration of approximately 1x10^6 CFU/ml. Cells were harvested from 1 ml of the bacterial suspension by centrifugation at 800×g for 15 min, and the bacterial cells were suspended in an equal volume of 90% serum at 37°C for 3 h. Complement activity was inactivated in control samples by heating at 56°C for 30 min. The sensitized bacterial samples were diluted 10-fold and plated onto DSA plates or LB plates, following which the number of viable cells was determined by direct colony counts on DSA agar. All assays were conducted in triplicate for *P. multocida* strains and *E. coli* DH5α. Serum sensitivity between the *P. multocida* P-1059 ∆*hexB* and P-1059C were compared for statistical significance using the Student's t-test.

**Protection assay**

Line 22 white Leghorn chickens (Verial Vital Laboratory Animal Technology Co. Ltd., China), approximately 56-day-old were used. Chickens were divided into 4 groups based on the strains for challenge-exposure (Table 6). Groups 1 and 3 were vaccinated with a live vaccine as an experiment groups while groups 2 and 4 were vaccinated with a sterile BHI broth as negative groups. Chickens of groups 1 and 3 were vaccinated with a single dose of a live vaccine with the concentration of 3.6x10^8 CFU/ml. Chickens were challenge-exposed at two weeks post vaccination. Chickens of groups 1 and 2 were challenge-exposed with 4.5x10^8 CFU/ml of the parent strain P-1059 to determine the homologous protection, while groups 3 and 4 were challenge-exposed with 3.7x10^8 CFU/ml of strain to determine the heterologous protection. The birds were observed for their mortality rates and clinical signs for ten days.

**Results**

**Cloning and sequencing of the *hexB* gene and its flanking sequence**

As shown in figure 1, a 2.9 kb fragment was successfully amplified from the genomic DNA of P-1059 by PCR. The PCR product was cloned into the pMD18-T vector and the nucleotide sequence of the inserts was determined. The DNA fragment was 2909 bp in length comprising three ORFs representing the three capsule transport protein genes *hexCBA*. The *hexC* contains 1137 nucleotides and terminates at a TAA stop codon, encoding a putative protein of 378 amino acids. The third base of the stop codon at the 3’-end of *hexC* is the first base of the ATG at the start of *hexB*, 798 nucleotides in length and coding for a putative protein of 265 amino acids. The *hexB* terminates with a TGA stop codon where it overlaps with *hexA*, the nucleotides ATGA forming part of the start codon. The *hexA* containing 660 nucleotides, encoding a putative protein of 220 amino acids and terminates at a TAA stop codon. The DNA homology of the *hexABC* genes between the *P. multocida* P-1059 and the previous reported *P. multocida* X-73 in GenBank was 99%.

**Construction of a targeting vector pWSK29∆hexB**

The targeting vector pWSK29∆hexB was designed to delete the *hexB* gene encoding capsular hyaluronic acid export protein B in *P. multocida* P-1059 by homologous recombination. The sequence analysis of targeting vector pWSK29∆hexB confirmed the presence of 1191 bp tetracycline resistant gene, the 991 bp *hexB* gene upstream fragment and the 976 bp *hexB* gene downstream segment of the *hexB* gene (data not shown). This indicates that the targeting vector pWSK29∆hexB was successfully constructed.

**Construction of *hexB* deletion mutant**

The targeting vector pWSK29∆hexB was transformed to *P. multocida* P-1059 by electroporation. By homologous recombination, *Ter* gene replaced *hexB* gene. Thus the *hexB* deletion mutant was generated. Several colonies exhibiting tetracycline resistance phenotype and growing on the DSA plate (containing 5 µg/ml tetracycline) were picked, and the putative mutant strains were screened by colony PCR. As shown in figure 2, using the primers P687F and P687R, a 687 bp fragment was amplified from the genomic DNA of parent strain P-1059. In contrast, no product was amplified from the genomic DNA of two putative mutants. These results indicated that the *hexB* gene was deleted from the genomic DNA of these strains. The *hexB* deletion strain was designated ∆*hexB*.
PCR analysis of mutant locus

The genomic DNAs from *P. multocida* P-1059 and ΔhexB were prepared, respectively. PCR primers P991F and P976R were used to amplify the expected 2.7-kb fragment from the genomic DNA of parent strain P-1059. The PCR results showed that a 3.2 kb fragment was amplified from the genomic DNA of ΔhexB. In constructing the targeting vector pWSK29ΔhexB, a 1191-bp tetracycline resistant gene was replaced with the 798 bp gene, accounting for the 393-bp difference in these PCR products (Figure 3). The sequences of the PCR products were cloned and confirmed by DNA sequencing.

RT-PCR analysis of hexB deletion mutant

The expression of the *hexB* was analyzed by RT-PCR of total RNA, using primers P687F and P687R. RT-PCR analysis showed that the expected 687-bp product was present in the parent strain P-1059 but was absent in the mutant ΔhexB (Figure 4). These results confirmed that the *hexB* gene was successfully deleted by homologous recombination. Complementation of the ΔhexB mutant with plasmid pPBA1101-hexB restored the *hexB* transcript to ΔhexB (Figure 4).

Observation of capsular structure of *P. multocida* strains

*P. multocida* strains were cultured on the DSA plates at 37°C for 18 h. Parent strain P-1059 produced mucoid colonies, consistent with the presence of a capsule, while both the mutant ΔhexB and the complemented strain P-1059C produced nonmucoid and small colonies (data not shown). As shown in figure 5, the capsule of mutant ΔhexB was thinner than that of the parental strain P-1059 according to electron microscopy. On the other hand, the complemented strain P-1059C had a thin and irregular capsule on bacterial cell surface.

Hyaluronic acid capsule production

The production of extracellular polysaccharide was determined by direct chemical assay for hyaluronic acid (Table 3). The viability of the cells was determined after the capsule extraction procedure and ranged from 10% to 30% of that prior to extraction. Mutant strain ΔhexB produced significantly less hyaluronic acid than did the parent strain P-1059 and complemented strain P-1059C (P<0.01). No significant difference between the hyaluronic acid produced by strains P-1059 and P-1059C (P>0.05). These results suggested that the complemented strain P-1059C had restored the ability to transport extracellular hyaluronic acid.

Virulence of *P. multocida* strains in chickens

The results of virulence test of *P. multocida* strains are shown in table 4. The parent strain P-1059 killed all chickens by intravenous injection at a dose of 10^5 to 10^7 CFU. In contrast, no deaths were recorded for chicken intravenous injection with 10^2 to 10^3 CFU of the mutant ΔhexB. However, the mutant ΔhexB revealed 60% and 100% mortality at high dose of 10^6 CFU and 10^7 CFU by intravenous injection, and the 50% lethal dose (LD50) was calculated to be approximately 5.14×10^7 CFU. On the other hand, the complemented strain P-1059C killed all chickens by intravenous injection at a dose of 10^5 to 10^6 CFU, whereas low doses of 10^3 and 10^4 CFU of the complemented strain P-1059C resulted in 20% and 60% mortality, and the LD50 was calculated to be approximately 6.71×10^7 CFU. These results show that the *hexB* deletion mutant is highly attenuated for virulence. Bacterial isolation was positive in all the dead chickens but negative in the surviving chickens.

Serum sensitivity assays

*P. multocida* strains P-1059, ΔhexB and P-1059C were incubated in 90% chicken serum to determine their resistance to complement-mediated killing. While a serum-sensitivity control of *E. coli* DH5α.
The growth of heat inactivity chicken serum. DH5α. No statistically significant differences were observed between was observed for the complemented strain P-1059C, increasing from 2.8×10^6 to 2.6×10^8 over 3 h of treatment. A similar trend killed in chicken serum, and the average number of CFU per milliliter decreased from 2.9×10^6 to 2.3×10^3 over 3 h of incubation. Heat inactivated serum permitted in the serum. As shown in table 5, the parent strain P-1059 was not was also examined to confirm the presence of complement activity in chickens. As shown in table 5, the parent strain P-1059 was not killed in chicken serum, and the average number of CFU per milliliter increased from 2.8×10^4 to 2.6×10^4 over 3 h of treatment. A similar trend was observed for the complemented strain P-1059C, increasing from an initial average of 3.2×10^4 to 3.6×10^4 CFU/ml over 3 h in chicken serum. However, the mutant ΔhexB was killed in chicken serum, and the average number of CFU per milliliter decreased from 2.9×10^6 to 2.3×10^5 over 3 h of incubation. Heat inactivated serum permitted growth of P. multocida strains P-1059, ΔhexB, P-1059C and E. coli DH5α. No statistically significant differences were observed between the bacteria number of P-1059, ΔhexB and P-1059C, after incubation in heat inactivity chicken serum.

Protection assay

Vaccines and results are shown in table 6. Chickens in group 1 was challenge-exposed with the parent strain P-1059, and complete protection (100% survivor) was obtained. Chickens in group 3 was challenge-exposed with heterologous wild-type strain of X-73 (A:1), highly protection (80% survivor) was obtained.

Discussion

The entire capsule locus of avian P. multocida X-73 (A:1) was cloned and sequenced, and the locus was divided into three regions, the region 1 of which contains four genes, hexD, hexC, hexB and hexA are predicted to encode proteins responsible for transport of the polysaccharide to the bacterial surface [11]. The sequence analysis demonstrated that the P. multocida hexABCD were highly homologous at both nucleotide and amino acid levels to Haemophilus influenzae hexABCD [18], Actinobacillus pleuropneumoniae cpxABCD [19] and Neisseria meningitidis ctrABCD [20]. In the serotype A:1 strain X-73, inactivation of the capsule transport gene hexA resulted in a mutant strain that was highly attenuated in both mice and chickens, and was more sensitive to the bactericidal activity of chicken serum [12].

Our previous study reported a noncapsulated mutant P-1059B obtained from 35 serial passages of P. multocida strain P-1059, demonstrated that the loss of ability to produce capsular materials resulted in a marked loss of virulence [7]. However, this study used spontaneously arising noncapsulated mutant. Thus in this study, we have constructed a hexB deletion mutant in the serotype A:3 strain P-1059, designated ΔhexB, and the ΔhexB was observed to be nonnucoid colony, and the cells of ΔhexB appeared acapsular by electron microscopy compared to the parent strain P-1059. An intact copy of hexB in the E. coli-P. multocida shuttle vector pPBA1101 was introduced into the mutant ΔhexB to complement the deleted hexB, with resultant strain designated P-1059C, and the P-1059C revealed a thick capsule material only on some cells. According to the hypothesis of previous report [12], the amount of extracellular capsule produced may not reflect its distribution on the surface of the cell. This result demonstrated that the hexB gene of P. multocida type A strain was responsible for transport of the polysaccharide to the bacterial surface.

Previous study reported the capsule as a virulence factor for chickens in an fowl cholera-causing P. multocida serotype A:1 strain by using a defined acapsular mutant [12]. In this study, the capsule was shown to be a virulence factor for P. multocida serotype A:3 strain in the chickens, by using the hexB deletion mutant. The acapsular mutant ΔhexB low virulence at a high dose as compared with the parent strain P-1059. When the intact hexB gene was restored in the complemented strain P-1059C, the ability to cause lethal infection was restored approximately to parent strain P-1059 levels in chickens. These results confirm previous work that capsule is major virulence factor in the pathogenesis of fowl cholera and show specifically that capsule is a critical virulence factor in the serotype A:3 strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Injected dose (CFU)</th>
<th>Mortality (%)</th>
<th>Bacterial isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1059</td>
<td>4.63 × 10^1</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4.63 × 10^2</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4.63 × 10^3</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4.63 × 10^4</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td>ΔhexB</td>
<td>6.46 × 10^4</td>
<td>0/5 (0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.46 × 10^5</td>
<td>0/5 (0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.46 × 10^6</td>
<td>0/5 (0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.46 × 10^7</td>
<td>3/5 (60)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6.46 × 10^8</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td>P-1059C</td>
<td>2.58 × 10^5</td>
<td>1/5 (20)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.58 × 10^6</td>
<td>3/5 (60)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.58 × 10^7</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.58 × 10^8</td>
<td>5/5 (100)</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>TB</td>
<td>0/5(0)</td>
<td>-</td>
</tr>
</tbody>
</table>

* CFU is colony forming units determined by viable count, # number of dead birds per five birds,

<table>
<thead>
<tr>
<th>Challenge strain (serotype)</th>
<th>Survival after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1059 (A:3)</td>
<td>5/5</td>
</tr>
<tr>
<td>P-1059 (A:3)</td>
<td>0/5</td>
</tr>
<tr>
<td>X-73 (A:1)</td>
<td>4/5</td>
</tr>
<tr>
<td>X-73 (A:1)</td>
<td>0/5</td>
</tr>
</tbody>
</table>
The result of serum resistance was consistent with a role for the serotype A capsule in survival in vivo. The parent strain P-1059 and complemented strain P-1059C were resistant to the bactericidal action of chicken serum, while acapsular mutant ∆hexB was highly sensitive. This agrees with previous results obtained by using spontaneously derived acapsular mutant and enzymatic removal of capsule [21] or using a defined acapsular mutant PBA930 [12]. These results demonstrated that the capsule of P. multocida serotype A:3 strains was responsible for protection against the bactericidal activity of complement, and the ability of ∆hexB to induce protection against both homologous and heterologous wild-type strains was similar to that of the acapsular mutant PBA930 [22].

In conclusion, we successfully constructed a genetically defined acapsular mutant of a serotype A:3 strain by homologous recombination. It was shown that deletion of the hexB gene resulted in the loss of surface-expressed capsular polysaccharide in this mutant. In chicken serum, the mutant ∆hexB was killed to a greater degree than the parent strain P-1059, indicating that the capsule of the P. multocida serotype A:3 strain is mediating resistance to serum bacteriolysis through the classical complement pathway. Moreover, the hexB deletion mutant is highly attenuated for virulence [23,24].

Acknowledgments

The present study was supported by the National Natural Science Foundation of China (30972206). We are grateful to Professor Ben Adler at Monash University for providing the plasmid pPBA1101 and the excellent technical assistance.

References