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Construction and Characterization of an Acapsular Mutant of Pasteurella multocida Strain P-1059 (A:3)

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Abstract

To further investigate the role of capsule involved in virulence of *Pasteurella multocida* P-1059 (A:3), a *hexB* deleted mutant was constructed by homologous recombination. The DNA replacement was confirmed by PCR, Reverse transcription (RT)-PCR and DNA sequencing. Experiments were conducted to compare the differences of biological characteristics such as capsular structure, capsular polysaccharide content, virulence and serum resistance between the *hexB* deleted mutant of Δ*hexB* and wild-type strain P-1059, as well as the complemented strain P-1059C. And the ability of the acapsular mutant Δ*hexB* to induced protection against wild-type challenge in chickens. Electron microscopy examination of the Δ*hexB* showed the absence of capsular material compared to the P-1059 and P-1059C. The Δ*hexB* was sensitive to the bactericidal action of chicken serum, whereas the P-1059 and P-1059C were both resistant. The Δ*hexB* was highly attenuated in chickens by intravenously injection, and intramuscular administration of Δ*hexB* to chickens stimulated significant protection against P-1059 and the homologous strain X-73(A:1). These results demonstrated that the capsule is a major virulence factor of *Pasteurella multocida* serotype A:3 strains.

Keywords: *Pasteurella multocida*; Homologous recombination; Knockout; Capsule; Virulence; Vaccine

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.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in table 1.

Strain or plasmid	Relevant features	References
Strains		
E. coli DH5α	*F⁻ endA1 hsdR17* (r_K^- m_K^+) *thi-1 λ⁻ recA1 Φ80dlacZΔM15*	TaKaRa
P. multocida		
X-73	Serotype A:1 wild-type strain	ATCC
P-1059	Serotype A:3 wild-type strain	ATCC15742
ΔhexB	The *hexB* deletion mutant of P-1059	This study
P-1059C	Δ*hexB* complemented with plasmid pPBA1101-*hexB*	This study
Plasmids		
pMD18-T	Cloning vector; Amp^r	TaKaRA
pBR322	Cloning vector Amp^r, Tet^r	TaKaRA
pWSK29	Low-copy-number *E. coli* cloning vector, Amp^r	[24]
pPBA1101	*E. coli-P. multocida* shuttle vector, Kan^r	[23]
pMD18-*hexABC*	The *hexB* gene and its flanking region was cloned into pMD18-T, Amp^r	This study
pWSK29Δ*hexB*	pWSK29 containing *Tet*ʳgene, and flanking region of the *hexB* gene; Amp^r, Tet^r	This study
pPBA1101-*hexB*	pPBA1101 containing the *hexB* gene, Kan^r	This study

Table 1: Bacterial strains and plasmids used in this study.

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Escherichia coli DH5α containing recombinant plasmid was cultured in LB-broth (Difco, USA) or on LB agar plate at 37°C. *P. multocida* strains were cultured in tryptose broth (Difco, USA) or on Dextrose starch agar (Difco, USA) at 37°C. When required, broth or agar was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml) or tetracycline (10 μg/ml for *E. coli* and 5 μg/ml for *P. multocida*).

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

For the PCR amplification, two specific primers P-1 (5′-ATGATC-GAAACAAAAATAC-3′) and P-2 (5′-CCCTATTCTTATTTACATG-3′) were designed according to the published *hexB* gene and its flanking sequence of *P. multocida* X-73 [11]. The expected size of the resulting amplicon was 2909 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-*hexABC*. The construct 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 pWSK29Δ*hexB*

The PCR primers were designed according to the sequenced *hexB* gene and its flanking sequence of *P. multocida* P-1059 (GenBank accession number JX679409) by using the primer premier 5.0 software program. The nucleotide sequences of these primers are described in table 2. The tetracycline resistance (Tet^r) gene was amplified from pBR322 plasmid by PCR using primers Tet-F and Tet-R (Table 2), and digested with *Sma* I/*Xho* I and inserted into the same restriction sites of the plasmid pWSK29 to generate pWSK29-*Tet^r*. 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^r* to generate the targeting vector pWSK29Δ*hexB*.

Construction of *P. multocida hexB* deletion mutant

Competent cells were prepared according to the previously described method [13] with slight modification. A 100 ml culture of *P. multocida* P-1059 cells was grown in TB medium to midlog phase and treated with 100 units/ml of hyaluronidase (Sigma, USA) for 1 h to remove capsule. The bacterial cells were harvested by centrifugation at 4000 × g for 15 min, and washed with sterile water for three times at 4°C. The bacterial pellet was suspended in 1 ml ice-water and placed on ice. The competent cells (40 μl) were mixed with 10 μg of the targeting vector pWSK29Δ*hexB* in 0.1 cm electroporation cuvette (Bio-Rad). Immediately after adding DNA, the cells were electroporated (Gene Pulser, Bio-Rad) at 2.5 kV, 25 μF, 600Ω with resultant time constants ranging 11 to 15 ms. 1 ml TB medium was added to the electroporated cells and the cells were then recovered at 37°C for 3 h, and plated onto DSA plates containing 5 μg/ml of tetracycline. Colonies were visible after 48 h incubation at 37°C, and the tetracycline resistance colonies were screened by colony PCR for the presence or absence of the *hexB* gene in the genomic DNA of putative mutant strains using primers P687F and P687R (Table 2).

Name	Sequence	Restriction site	PCR product
P991F	5′-CGCTCTAGATTTCTCAATCGAGTTTTGTTGT-3′	*Xba* I	991 bp
P991R	5′-CGCCCCGGGTAATTTTTATGCTCTCGAATGC-3′	*Sma* I	
P976F	5′-CGCCTCGAGTTTATGTAGAAAACGTCTGT-3′	*Xho* I	976 bp
P976R	5′-GCGGGTACCCCCTATTCTTATTTACATG-3′	*Kpn* I	
Tet-F	5′-GCGCCCGGGATGAAATCTAACAATGCGCTCATCG-3′	*Sma* I	1191 bp
Tet-R	5′-CGCCTCGAGTCAGGTCGAGGTGGCCCG-3′	*Xho* I	
P687F	5′-ATAGAGTGATTGGTGCTCTC-3′		687 bp
P687R	5′-TTAAGTCACATAAAATGAGG-3′		
HexB-F	5′-CGCGAGCTCATGTTATACGATGACC-3′	*Sac* I	798 bp
HexB-R	5′-CGCTCTAGATCATCGAGGTTCTATC-3′	*Xba* I	

Table 2: Oligonucleotide primers used in this study.

PCR analysis of mutant locus

After replacing the *hexB* gene in *P. multocida* P-1059 by the *Tet^r* gene in targeting vector pWSK29Δ*hexB*, the *hexB*-deleted strain was obtained. The mutant locus in genomic DNA of this mutant was confirmed by PCR using primer pairs P687F and P687R, P991F and P976R, respectively. Simultaneously, the PCR products were cloned into the pMD18-T vector using *E. coli* DH5α. The sequence of the inserts was determined to confirm mutant locus.

Complementation of *hexB* deletion mutant

The entire *hexB* gene was amplified from genomic DNA of *P. multocida* P1-59 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 *E. coli-P. multocida* shuttle vector pPBA1101, generating pPBA1101-*hexB*, which was introduced into the *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 (Tiangen, China) according to the manufacturer's instructions. The primers used for RT-PCR analysis were P687F and P687R (Table 2). PCRs on the RT product and no RT control were performed with *Ex Taq* polymerase (TaKaRa, China) using standard procedures.

Observation of capsular structure of *P. multocida* strains

Bacterial cells were prepared for transmission electron microscopy as described previously [14]. Briefly, *P. multocida* strains were inoculated into fresh TB medium and incubated at 37°C for 6 h. A 250 μl aliquot of the broth culture was spread on DSA plates and incubated at 37°C for 18 h. The bacteria grown on the DNA plate were suspended in 0.1 M cacodylate buffer containing 5% glutaraldehyde and 0.15% ruthenium red for fixation and staining, respectively. The bacterial suspension was incubated at room temperature for 2 h, and the bacteria were collected by centrifugation at 480 × g for 10 min. The bacterial cells were suspended in 0.05 M cacodylate buffer and allowed to react with a 1.0 mg/ml polycationic ferritin (Sigma) at room temperature for

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 tetraoxide for 1 h. The specimens were dehydrated in graded ethanols, 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 microcopy 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, Δ*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 with aeration until mid-exponential phase (OD_{600} of ~0.5). Cells were harvested from 5 ml of bacterial culture by centrifugation at 7,600 × g for 15 min, washed once with sterile phosphate-buffered saline (PBS; pH7.4), 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, Δ*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 1×10^6 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 inactived 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 Δ*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 3.6×10^8 CFU/ml. Chickens were challenge-exposed at two weeks post vaccination. Chickens of groups 1 and 2 were challenge-exposed with 4.5×10^3 CFU/ml of the parent strain P-1059 to determine the homologous protection, while groups 3 and 4 were challenge-exposed with 3.7×10^3 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 pWSK29Δ*hexB*

The targeting vector pWSK29Δ*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 pWSK29Δ*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 pWSK29Δ*hexB* was successfully constructed.

Construction of *hexB* deletion mutant

The targeting vector pWSK29Δ*hexB* was transformed to *P. multocida* P-1059 by electroporation. By homologous recombination, Tet^r 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 Δ*hexB*.

PCR analysis of mutant locus

The genomic DNAs from *P. multocida* P-1059 and Δ*hexB* 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 Δ*hexB*. In constructing the targeting vector pWSK29Δ*hexB*, a 1191-bp tetracycline resistant gene was replaced with the 798 bp *hexB* 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 Δ*hexB* (Figure 4). These results confirmed the *hexB* gene was successfully deleted by homologous recombination. Complementation of the Δ*hexB* mutant with plasmid pPBA1101-*hexB* restored the *hexB* transcript to Δ*hexB* (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 Δ*hexB* and the complemented strain P-1059C produced nonmucoid and small colonies (data not shown). As shown in figure 5, the capsule of mutant Δ*hexB* 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 Δ*hexB* 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 10^1 to 10^5 CFU. In contrast, no deaths were recorded for chicken intravenous injection with 10^4 to 10^6 CFU of the mutant Δ*hexB*. However, the mutant Δ*hexB* revealed 60% and 100% mortality at high dose of 10^7 CFU and 10^8 CFU by intravenous injection, and the 50% lethal dose (LD_{50}) 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 10^7 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 LD_{50} was calculated to be approximately 6.71×10^3 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.

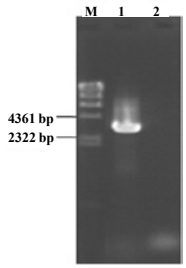

Figure 1: PCR amplification of *hexB* gene and its flanking region from *P. multocida* P-1059 using primers P-1 and P-2. lane M: λ-*Hind* digest DNA marker, lane 1: PCR product, lane 2: Negative control.

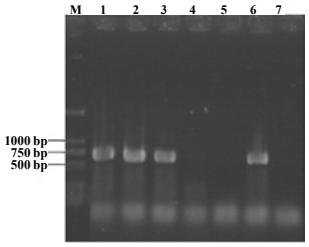

Figure 2: Identification of *hexB* mutants by colony PCR using the primers P687F and P687R. Lane M: DL2000 DNA marker, lane 2: PCR product of P-1059, lanes 2, 3 and 6: PCR-positive colonies, lanes 4 and 5: PCR-negative colonies, lane 8: Negative control.

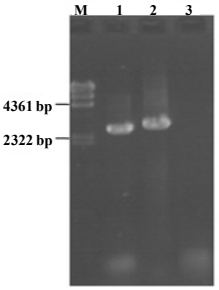

Figure 3: PCR verification of *hexB* deletion mutant of ΔhexB using the primers P991F and P976R. Lane M: λ-*Hind*III digest DNA marker, lane 1: PCR product of parent strain P-1059, lane 2: PCR product of mutant ΔhexB, lane 3: negative control.

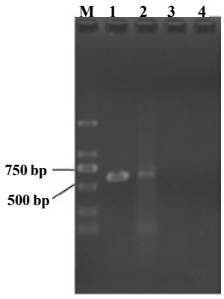

Figure 4: RT-PCR analysis of *P. multocida* strains using primers P687F and P687R. Lane M: DL2000 DNA marker, Lane 1: total RNA of P-1059, Lane 2: total RNA of P-1059C, Lane 3: total RNA of ΔhexB, Lane 4: Negative control.

Serum sensitivity assays

P. multocida strains P-1059, Δ*hexB* 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

P. multocida strain	Amounts of hyaluronic acid (10^{-8} μg/CFU)[a]	Viability[b]
P-1059	12.2 ± 0.9	0.2 ± 0.1
Δ*hexB*	2.3 ± 0.3	0.4 ± 0.3
P-1059C	11.4 ± 0.6	0.1 ± 0.2

[a] The levels of hyaluronic acid produced by strains P-1059 and P-1059C compared to that of Δ*hexB* were significantly

different (P<0.01), [b]Viability is expressed as the ratio of bacterial number (CFU/ml) after treatment at 42°C to that before treatment

at 42°C.

Table 3: Extracellular hyaluronic acid produced by *P. multocida* strains.

Strain	Injected dose (CFU)[a]	Mortality[b] (%)	Bacterial isolation
P-1059	4.63×10^1	5/5 (100)	+
	4.63×10^2	5/5 (100)	+
	4.63×10^3	5/5 (100)	+
	4.63×10^4	5/5 (100)	+
	4.63×10^5	5/5 (100)	+
Δ*hexB*	6.46×10^4	0/5 (0)	-
	6.46×10^5	0/5 (0)	-
	6.46×10^6	0/5 (0)	-
	6.46×10^7	3/5 (60)	+
	6.46×10^8	5/5 (100)	+
P-1059C	2.58×10^3	1/5 (20)	+
	2.58×10^4	3/5 (60)	+
	2.58×10^5	5/5 (100)	+
	2.58 × 106	5/5 (100)	+
	2.58×10^7	5/5 (100)	+
Control [c]	TB	0/5(0)	-

[a] CFU is colony forming units determined by viable count, [b] number of dead birds per five birds,

[c] Nontreated control chickens.

Table 4: Virulence of *P. multocida* strains in chickens.

was also examined to confirm the presence of complement activity in the serum. 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 2.8×10^6 to 2.6×10^8 over 3 h of treatment. A similar trend was observed for the complemented strain P-1059C, increasing from an initial average of 3.2×10^6 to 3.6×10^7 CFU/ml over 3 h in chicken serum. However, the mutant Δ*hexB* was 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 growth of *P. multocida* strains P-1059, Δ*hexB*, P-1059C and *E. coli* DH5α. No statistically significant differences were observed between the bacteria number of P-1059, Δ*hexB* 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 influenza bexABCD* [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 Δ*hexB*, and the Δ*hexB* was observed to be nonmucoid colony, and the cells of Δ*hexB* 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 Δ*hexB* 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 Δ*hexB* 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.

Strains	Serum heat treatment	Survival ratio
E. coli DH5α	-	0[a]
	+	8.6[a]
P. multocida		
P-1059	-	165 ± 57
	+	196 ± 64
Δ*hexB*	-	0.24 ± 0.07[a]
	+	126 ± 22[a]
P-1059C	-	97 ± 19[a]
	+	142 ± 26[a]

[a] Significantly different at P<0.01 when compared to serum and heated serum treatment

Table 5: Resistance of *E. coli* DH5 and *P. multocida* strains to chicken serum.

Group of chicken	Challenge dose (CFU)	Challenge strain (serotype)	Survival after challenge
1	4.5×10^3	P-1059 (A:3)	5/5
2	BHI broth	P-1059 (A:3)	0/5
3	3.7×10^3	X-73 (A:1)	4/5
4	BHI broth	X-73 (A:1)	0/5

Table 6: Protection confirmed in chickens by immunization of live *P. multocida* ΔhexB against challenge with *P. multocida* strains.

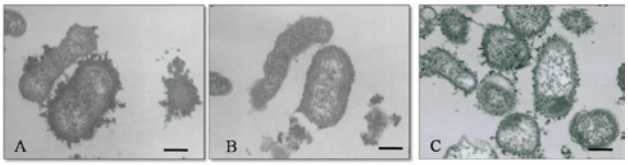

Figure 5: Electron micrographs of *P. multocida strains* P-1059 (A), P-1059C (B) and ΔhexB (C). Bars, 200 nm.

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 resistance to the bactericidal action of chicken serum, while acapsular mutant Δ*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 Δ*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 Δ*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].

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