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Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat
Molecular Characterization of an Adhesive Protein of *Pasteurella multocida* Strain P-1059 and its Variant Strain P-1059B

Entomack Borrathybay1),*, Nattawooti Sthitmatee1),**, Kenichi Suzuki1), Ryo Shinnakasu1), Shuichi Tsuchida2), Ryozo Akuzawa3), Yasushi Kataoka1) and Takuo Sawada1)

1) Laboratory of Veterinary Microbiology, Nippon Veterinary and Life Science University
2) Comparative Cellular Biology, Nippon Veterinary and Life Science University
3) Animal Products Science and Technology, Nippon Veterinary and Life Science University
* Present address : College of Biology and Environmental Sciences, Jishou University, PR China
** Present address : Faculty of Veterinary Medicine, Chiang Mai University, Thailand

Abstract

The adhesive and cross-protective 39 kDa protein (Cp39) antigen of *Pasteurella multocida* capsular serogroup A strain P-1059 and its variant strain P-1059B was genetically identified and characterized. The predicted molecular mass of Cp39 of strain P-1059 was 37 kDa containing 353 amino acids with 20 amino acids signal peptide while the predicted molecular mass of Cp36 of variant strain P-1059B was 35 kDa containing 343 amino acids with 20 amino acids signal peptide. The *cp39* gene of strain P-1059 was identical to the major *ompH* gene of strain X-73 while the *cp36* gene of strain P-1059B was identical to the major *ompH* gene of strain P-1059. These results indicate some differences between *cp39* gene and *ompH* gene of strain P-1059. The recombinant Cp39 (rCp39) was produced by *Escherichia coli* and characterized. Rabbit antisera against Cp39, rCp39 and rOmpH showed the cross-reactions by Western blots analysis and significantly (*P< 0.01) inhibited adhesion of strains X-73, P-1059 and P-1059B to chicken embryo fibroblast (CEF) cells. These results definitely demonstrated that the rCp39 of strain P-1059 was a common antigen and a common adherence factor among *P. multocida* capsular serogroup A strains.


*Pasteurella multocida* strain of capsular serogroup A and somatic serotypes 1, 3 or 4 are recognized as the causative agent of fowl cholera5). Clinical signs range from mild upper respiratory tract infection to septicemia. The main route of infection is the mucosal surface of the respiratory tract. Extensive functional studies of the potent virulence factors have been carried out5, 10, 17, 18).

Our previous investigations have identified the significance of the 39 kDa capsular protein (Cp39) of *P. multocida* strain P-10595) Non-capsulated variant strain of *P. multocida* strain P-1059, designated as strain P-1059B, was obtained by 35 serial passages on dextrose starch agar and appeared bluish colony when observed under the obliquely-transmitted light stereomicroscope7). Strain P-1059B has low amounts of cap-
Molecular characterization of adhesive protein of avian *P. multocida*

Sular protein and they could not classify its serogroup by indirect hemagglutination (IHA) test. This variant strain lost of adherence ability to chicken embryo fibroblast (CEF) cells and was low virulent for chicken when compared to the encapsulated strain. However, the gene encoding Cp of strain P-1059 and genetic variation of the phenotypic non-encapsulated variant strain P-1059B were not determined yet.

Then, the present study was performed to clone and sequence the adhesive protein gene of strain P-1059 and compared to its variant strain P-1059B. Additionally the recombinant adhesive protein of strain P-1059 was prepared in *Escherichia coli* and compared immunologically to the major outer membrane protein H (OmpH) gene of strain X-73[15].

**Materials and Methods**

**Bacterial strains and plasmids.** The strains of *P. multocida* and *E. coli* used in this study are shown in Table 1. *P. multocida* strains were grown in tryptose broth (TB; Difco Laboratories, Detroit, MI, USA) at 37°C for 6 h and were then cultured on dextrose starch agar (DSA; Difco) at 37°C for 18 h. One single colony was selected for crude capsular extract (CCE) and for genomic DNA preparation. *E. coli* strain INVαF' (Invitrogen, Carlsbad, CA, USA), strain M15 [pREP4] (Qiagen, Valencia, CA, USA) and strain JM109 (Promega, Madison, WI, USA) were cultured at 37°C in Luria broth (LB) or on LB agar plates with the appropriate antibiotics. Plasmid pCR2.1 (Invitrogen) and plasmid pQE-30 (Qiagen) were used for cloning and recombinant protein expression.

**N-terminal amino acid sequencing of 39kDa protein.** Crude capsular extract (CCE) of *P. multocida* strain P-1059, P-1059B and X-73 were prepared according to the saline extraction method as described previously. The Cp39 protein was purified by electroelution method as described previously.[16] Purified Cp39 protein and recombinant proteins were separated by SDS-PAGE through 12.5% acrylamide slab gels. The proteins were then transferred to immunobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and subsequently subjected to Edman degradation in an applied PPSQ-21/23 protein sequenator (Hitachi, Tokyo, Japan).

**Amplification of the cp39 gene by PCR.** *P. multocida* genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) precipitation method. Amplification of the cp39 gene was performed with 20 ng of *P. multocida* genomic DNA; 30 pmol each of primer Cp39-F and Cp39-R (GenBank Accession No.EF203903) ; 0.1 mM dNTP, 1.5 mM MgCl2 and 1.25 units of Taq DNA Polymerase (Takara, Shiga, Japan) in a total volume 50 μl of reaction buffer. The

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Genotype or other relevant characteristics</th>
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<tr>
<td><strong>P. multocida</strong></td>
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<td></td>
</tr>
<tr>
<td>P-3827</td>
<td>Reference strain capsular serogroup A</td>
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</tr>
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<td>P-4679</td>
<td>Reference strain capsular serogroup F</td>
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<td>Serovar A:3, Iridescent colony</td>
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<tr>
<td>P-1059B</td>
<td>-3, Blue colony, capsular ungroupable</td>
<td>ATCC11039</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td>INVαF'</td>
<td>F'/ recA1,recA1,hsdR17(r3, m-, ) supE44,thi-1, gyrA96,relA1,pho80lacZΔM15,ΔlacZYA-argF1U169,λ'</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>M15[pREP4]</td>
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<td>Qiagen</td>
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<td><strong>Plasmids</strong></td>
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<td>pCR 2.1</td>
<td>TA cloning vector</td>
<td>Invitrogen</td>
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<td>pQE-30</td>
<td>N-terminal 6×His tag expression vector</td>
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<td>pQE-cp39</td>
<td>N-terminal 6×His tag expression vector contained cp39 gene of strain P-1059</td>
<td>This study</td>
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<tr>
<td>pQE-ompH</td>
<td>N-terminal 6×His tag expression vector contained ompH gene of strain X-73</td>
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amplification reactions were performed with GeneAmp PCR System 9700 (AB Applied Biosystems, Foster City, CA, USA) under following conditions: 94°C for 5 min; then 35 cycles each at 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min; and lastly 72°C for 10 min. PCR products were analyzed in 1.5% agarose gel electrophoresis (Sigma Aldrich, St. Louis, MO, USA) and stained with ethidium bromide. Pictures were taken under UV illuminator.

Cloning of PCR products and sequence determination. PCR products were purified using QIAquick PCR purification kit (Qiagen) and were ligated into pCR 2.1 vector according to the manufacturer’s instructions. The ligations were introduced by transformation into CaCl₂-treated *E. coli* strain INVαF. Blue and white colonies were screened on selective LB agar plates containing 100 μg/ml ampicillin (Sigma) and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal ; Takara). The presence of inserted DNAs in plasmids was identified by another PCR assay employing primer Cp-F and Cp-R with the reaction and condition as described above. The recombinant plasmids of pCR 2.1-cp39 were purified with the QIAprep Miniprep kit (Qiagen) according to the manufacturer’s instruction. Sequence determinations were employed with M13 forward and M13 reverse primer (Table 2) by dideoxy chain termination method with the SQ5500E DNA sequencer (Hitachi Electronics Engineering Co., Ltd., Tokyo, Japan). Sequence analysis was conducted with Hitachi DNAsis Pro 3.0 software (Hitachi Software Engineering Co., Ltd., CA, USA). Sequence similarity searches were performed at the National Center for Biotechnology Information with the BLAST network service.

Expression and purification of the recombinant protein. The encoding mature protein of *cp39* gene of strain P-1059 was amplified from recombinant plasmid DNA with primer 5 Cp-F and 3 Cp-R (Table 2). The purified PCR product was double-digested with *BamHI* and *XhoI* (Takara) and purified as described above. Purified *BamHI* and *XhoI* — digested *cp39* fragments were ligated into the *BamHI* and *SalI* — predigested expression vector pQE-30, and introduced into *E. coli* strain M15[pREP4] by transformation. Transformants were plated on selective LB agar plates containing 100 μg/ml ampicillin and 25 μg/ml kanamycin (Sigma). The plasmids of transformants were taken and sequenced by employing sequencing primer Type III/IV forward and reverse sequencing (Table 2). Transformants were cultured in LB broth with or without the induction of IPTG (isopropyl-β-D-thiogalactopyranoside ; Takara) at the final concentration of 1 mM. The recombinant protein could be easily purified by the affinity chromatography with Ni-nitrilotriacetic acid resin (Ni-NTA ; Qiagen). The concentration of recombinant Cp39 (rCp39) protein was determined by

Table 2. Primers used in this study

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<tr>
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<th>Sequence</th>
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<td>1-21</td>
</tr>
<tr>
<td>Cp39-R</td>
<td>5’-TTAGAAGTGTACCGTAAACC - 3’</td>
<td>1032-1012</td>
</tr>
<tr>
<td>5 Cp39-F</td>
<td>5’-CAGGATCCGCAACAGTTTACATCGAC - 3’</td>
<td>61-81</td>
</tr>
<tr>
<td>3 Cp39-R</td>
<td>5’-CACTCGAGTTAAGTGTACCGTAAACC - 3’</td>
<td>1032-1012</td>
</tr>
<tr>
<td>M13 forward</td>
<td>5’-TGTTAAAACGGCCGAGT - 3’</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>M13 reverse</td>
<td>5’-CAGGAAACAGCTATGAC - 3’</td>
<td>Invitrogen</td>
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<tr>
<td>Type III/IV forward</td>
<td>5’-CGGATAACAAATTCACACAG - 3’</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>Reverse sequencing</td>
<td>5’-GTTTCGGAGTCTACCTGAG - 3’</td>
<td>QIAGEN</td>
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a Two extra bases and *BamHI* restriction site were added at the 5’ end to facilitate subcloning of the PCR products.

b Two extra bases and *BamHI* restriction site were added at the 5’ end to facilitate subcloning of the PCR products.

c and d positions in the pCR2.1 cloning vector.

e and f positions in the pQE-30 expression vector.
Molecular characterization of adhesive protein of avian *P. multocida*

93

the Bradford assay\(^1\). Additionally, the recombinant OmpH (rOmpH) of strain X-73 was expressed and purified by using the *E. coli* expression system as described previously\(^{16}\).

**Preparation of rabbit antiserum.** Antisera against expressed proteins of rCp39 and rOmpH were prepared by immunizing the adult male Japanese White rabbits (Funabashi Animal Research Center, Chiba, Japan). Rabbits were subcutaneously injected with a 200 \(\mu\)g of protein emulsified in an equal volume of incomplete Freund's adjuvant (Sigma) and injection was repeated three times at 30-day intervals. Blood was collected at 14 days after the forth injection. Antisera were absorbed with *E. coli* strain M15 whole-cell lysates\(^{17}\) before prior to use for Western blots analysis or adhesion inhibition assay. Rabbit antiserum against purified Cpf39 of strain P-1059\(^{19}\) was also used in this study.

**SDS-PAGE and Western blotting.** Proteins were analyzed on a 12.5% polyacrylamide slab gel according to Laemmli's method\(^1\)\(^2\) in a mini-slab apparatus (ATTO Corporation, Tokyo, Japan) and stained with Coomassie blue R-250 (Sigma). Separated proteins were transferred to the nitrocellulose membranes (Amersham Biosciences KK, UK) by semi-dry system and immunostained with specific antiserum at dilution of 1 : 300 and with 1 : 1000 dilutions of the horseradish peroxidase-conjugated anti-rabbit IgG (Sigma). Lastly, transferred proteins were visualized using 20 mg/ml of 3,3'-diaminobenzidine (DAB; Sigma) in phosphate-buffered saline (PBS, pH 7.2) as a chromogenic substance.

**Adhesion inhibition assay.** Adhesion inhibition assay was performed according to the previously described method\(^7\). CEF cells were prepared from 9 to 10-day-old SPF embryonated chicken eggs (Line-M; Nippon Institute for Biological Science, Tokyo, Japan). Concentration of approximately 1 \(\times 10^6\) cfu/ml of strains X-73, P-1059 and P-1059B were prepared to determine the adhesion to CEF cells. To demonstrate the surface protein of avian *P. multocida* capsular serogroup A strains corresponding the adhesion to CEF cells, the bacterial suspensions were treated with rabbit antiserum against Cp39 of strain P-1059, or rCp39 of strain P-1059 or rOmpH of strain X-73. Additionally, treatments with normal rabbit serum or PBS were done as the control.

**Statistical analysis.** To quantify the adherence, the number of bacteria adhering to CEF cells was counted and the results were compared for statistical significance using the unpaired Student's t-test.

**Multiplex PCR capsular serogrouping.** Genomic DNA of each strain were prepared and used for capsular PCR typing. Set of primers is shown in Table 2. The capsular serogroups were determined by multiplex capsular PCR typing as described previously\(^{21}\).

**Results**

**N-terminal amino acid sequencing.** The primary Cp39 of strain P-1059 had a predicted molecular mass of 37 kDa, which totally contained 353 amino acids with 20 amino acids signal peptide. N-terminal amino acid of Cp39 of strain P-1059 was almost identical to that of previously reported OmpH of strain P-1059 and also similar to that of other reported putative protein of *P. multocida*\(^{14,16}\). The first 20 amino acid residues were determined. Homology between the 20 amino acid residues of Cp39 of strain P-1059 and OmpH of strain X-73 was 85% (Fig. 1).

**Cloning and sequencing of cp39 gene of *P. multocida*.** The cp39 gene was successfully amplified from genomic DNA of strain P-1059 and its variant strain P-1059B, respectively. The coding region cp39 gene of strain P-1059 was 1,062 bp in length (GenBank Accession No. EF203903). DNA sequences of the cp39 gene of strain P-1059 were identical to the major ompH gene of strain X-73 (GenBank Accession No. U50907). On the other hand, the coding region cp36 gene of P-1059B was 1,032 bp in length and identical to the major ompH gene of strain P-1059 (GenBank Accession No. EF203904). The mature protein contains 343 amino acids with a predicted molecular mass of 35 kDa. DNA homology between cp36 gene and cp39 gene was 84% (data not shown).

**Expression of recombinant Cp39. SDS-PAGE and Western blots.** Transformation of recombinant pQE-30, which contained the cp39 gene for mature protein without a signal peptide, resulted in about 200 colonies. One colony that carried the right sequence was chosen for further expression analysis and this colony was designated *E. coli* strain PQE-cp39. The rCp39 had 12 amino acids fused at the N-terminal of mature Cp39 without signal peptide. The rCp39 was analyzed on SDS-PAGE and detected by Western blots (Figs. 2–6). *E. coli* harboring pQE-cp39 produced the recombinant protein, while the uninduced *E. coli* harboring pQE-cp39 did not produce the rCp39. This indicated that the T5 promoter in pQE-cp39 tightly controlled and that the mature rCp39 was not toxic for *E. coli*. The fusion recombinant protein had a molecular mass of about 37 kDa (Fig. 2). Additionally, transformants of *E. coli* colonies, which carried out plasmid pQE-ompH containing...
Fig. 1. Comparison of N-terminal amino acid sequence alignment. N-terminal amino acid sequence alignment (amino acid residues 1–20) of Cp39 protein from P. multocida strain P-1059 with that of the OmpH protein of strain P-1059 (upper alignment) and OmpH protein of strain X-73 (lower alignment) using the one letter symbols for amino acids. Identical residues are indicated by continuous lines. Positions are numbered from the N-termini.

Fig. 2. SDS-PAGE of P. multocida strain, E. coli whole-cell lysate and purified recombinant protein on a 12.5% gel stained with Coomassie blue. Lanes: M, molecular mass standards; 1, whole-cell lysate of strain P-1059; 2, whole-cell lysate of IPTG induced and 3, IPTG uninduced clone of PQE-cp39; 4, whole-cell lysate of IPTG uninduced and 5, IPTG induced E. coli M15 harboring pQE30 without insert; 6, purified rCp39 of strain P-1059; 7, whole-cell lysate of strain X-73; 8, whole-cell lysate of IPTG induced and 9, IPTG uninduced clone PQE-ompH; 10, purified rOmpH of strain X-73.

Fig. 3. Western blot assay using rabbit antiserum against Cp39 of strain P-1059 to P. multocida strains, E. coli whole-cell lysate and purified recombinant protein. Lanes: M, molecular mass standards; 1, whole-cell lysate of strain P-1059; 2, whole-cell lysate of IPTG induced and 3, IPTG uninduced clone of PQE-cp39; 4, whole-cell lysate of IPTG uninduced and 5, IPTG induced E. coli M15 harboring pQE30 without insert; 6, purified rCp39 of strain P-1059; 7, whole-cell lysate of strain X-73; 8, whole-cell lysate of IPTG induced and 9, IPTG uninduced clone PQE-ompH; 10, purified rOmpH of strain X-73.

In Western blot analysis (Figs. 3–5), rabbit antisera against purified Cp39 and rCp39 of strain P-1059 reacted with both of the Cp39 and rCp39. In addition, rabbit
Molecular characterization of adhesive protein of avian *P. multocida*

antiserum against rOmpH of strain X-73 also reacted with both of Cp39 and rCp39 of strain P-1059. These demonstrated that rabbit antiserum reacted with the native and recombinant proteins and cross-reaction was observed among strains P-1059 and X-73.

**Adhesion inhibition assay by treatment with anti sera.** Influence of treatment of *P. multocida* capsulated strains X-73, P-1059 and its non-capsulated variant strain P-1059B with rabbit antiserum on their ability to adhere to CEF cells is shown in Fig. 6. Rabbit anti-Cp39, anti-rCp39 or anti-rOmpH sera-treated bacterial cells significantly reduced (*P* < 0.01) the number of adhered bacteria as compared to the treatment with normal rabbit serum or PBS.

**Multiplex PCR capsular serogrouping.** All reference strains of *P. multocida* showed their own PCR product pattern as described previously[20]. Capsular serogroup A strain showed 1,044 and 460 bp in size of PCR products. Strain P-1059 and its variant strain P-1059B also showed the same PCR product pattern as capsular serogroup A strain. This resulted that strain P-1059B could be categorized into *P. multocida* capsular serogroup A strain based on this serogrouping system. PCR products of all the isolates are shown in Fig. 7.

**Discussion**

Capsular protein is one of the virulent factors among the gram-negative bacteria including *P. multocida*. Ali et al.[9] demonstrated that a 39 kDa protein or capsular protein of 39 kDa (Cp39) was an adherence factor to CEF cells and purified with the affinity chromatography using a specific monoclonal antibody (Mab). The present study demonstrated that the amino acid sequence of the Cp36 was almost identical to the gene encoding of OmpH of *P. multocida* strain P-1059 (GenBank Accession No.U52200) while the Cp39 was also almost identical to the *ompH* gene of strain X-73 (GenBank Accession No.U50907). Differences between these genes were observed at first 20-amino-acid residues with 85% homology (Fig. 1). The previous studies indicated that the Cp39 of *P. multocida* was located in the outer membrane of bacterial cell[14,15]. In contrast, the Mab-based immunoelectron microscopy demonstrated that the Cp39 was located abundantly in the bacterial capsule and played roles as an adherence factor and a cross-reactive antigen among avian *P. multocida* capsular serogroup A strains of various somatic serotypes[9]. Our present results coupled with those of the previous studies[6,7,8,14,15] suggested that OmpH may be origin of Cp39 and may infiltrate into
capsule from outer membrane, and work as an adherence factor inducing higher pathogenicity of encapsulated *P. multocida*.

Homology of gene encoding Cp between strains P-1059 and P-1059B was 84% (data not shown). The gene coding Cp39 was identical to the *ompH* gene of strain X-73 (GenBank Accession No.U50907) while the *cp36* gene of strain P-1059B was identical to the *ompH* gene of *P. multocida*. 

Fig. 6. Influence of sensitization with rabbit antisera of capsulated strain X-73, P-1059 and its variant strain P-1059B on their adherence to CEF cells. ** significantly different at *P* < 0.01 when compared to the PBS or NS treatments. a**: significantly different at *P* < 0.01 when compared to the capsulated strain P-1059. PBS: phosphate buffered-saline; NS: normal rabbit serum; 39KDS: rabbit antiserum against native Cp39 of strain P-1059; Cp39S: rabbit antiserum against rCp39 and OmpHS: rabbit antiserum against rOmpH.

Fig. 7. Multiplex PCR for capsular typing of *P. multocida* strain. Lane : M, 100bp DNA ladder (Takara); 1, mixed DNA of all capsular type A, B, D, E and F; 2, capsular type A strain P-3827; 3, capsular type B strain P-1256; 4, capsular type D strain 3881; 5, capsular type E strain P-1235; and 6, capsular type F strain P-4679; 7, strain P-1059; and 8, strain P-1059B, respectively.

Fig. 8. SDS-PAGE 12.5% gel stained with Coomassie blue of crude capsular extract (CCE) of *P. multocida* strains and recombinant proteins. Lane M, molecular mass standard; Lane 1, strain P-1059B; Lane 2, strain P-1059; Lane 3 and 4, purified rCp39; Lane 5, CCE of strain X-73; Lane 6 and 7, purified rOmpH.
multocida strain P-1059 (GenBank Accession No. U 52200). This corresponded that the major protein of strain P-1059B was approximately 36 kDa on SDS-PAGE and encoded with the cp36 gene (Fig. 8). In addition, this suggested that the variation of the cp39 gene into the cp36 gene of strain P-1059B corresponded to its decreased adhesion to CEF cells when compared to the encapsulated strain P-1059. The bluish colonies of strain P-1059B was derived from strain P-1059 by 35 serial passages on DSA as described previously. Borrathbybay et al.5,7 demonstrated that the low amounts of the 39 kDa capsular protein of strain P-1059B correlated to its low adhesiveness for chickens still remained. However, capsular serogroup of strain P-1059B could not be identified by the IHA test6 while multiplex PCR could. Multiplex PCR product pattern of strain P-1059B indicated that strain P-1059B could be categorized into capsular serogroup A strain by this method even though the low amount of capsular polysaccharide resulted untypable in the IHA-based capsular grouping.

The recombinant Cp39 expressed in E. coli indicated that the T5 promoter in pQE-cp39 was strongly controlled and derived mature recombinant protein was not lethal for E. coli. The cross-reaction of rabbit antiserum against rCp39 to P. multocida capsular serogroup A strains were similar to the cross-reaction of rabbit antiserum against Cp3910. This correlated to the homology between the Cp39 of strain P-1059 and OmpH of strain X-73 as described above. The bacterial cells of P. multocida strains P-1059, P-1059B and X-73 treated with rabbit antiserum against Cp39 or rCp39 of strain P-1059 showed a significant reduction (P<0.01) in the number of adhered bacteria to CEF cells, indicating that the antibodies might block the interaction between the Cp39 and CEF cells receptor, which resulted in inhibition of the adhesion. Moreover, rCp39 also mediated the adhesion of capsular serogroup A strain to CEF cells similar to its Cp39 as described in our previous study7. In addition, the amino acid sequence of the Cp39 of strain P-1059 also shared a high homology with the 35 kDa protein of the major outer membrane protein of capsular serogroup A strain isolated from cattle 1(data not shown). Lübbe et al.10 also indicated that 35 kDa protein from bovine isolate is an adherence factor of the organism, after treatment of such adhesion with specific antibodies prepared against this protein. Likewise, it was demonstrated by a specific Mab against the Cp39 of strain P-1059 that the Cp39 was an adherence factor of P. multocida capsular serogroup A strain to CEF cells8.

In conclusions, the gene encoding Cp39 of strain P-1059 and its variant strain P-1059B was successfully clarified. The cp39 gene of strain P-1059 and cp36 gene of strain P-1059B were cloned and sequenced. The recombinant protein of Cp39 was expressed in E. coli and the adhesive capacity of the protein was assessed. The results confirmed that the Cp39 protein of avian P. multocida capsular serogroup A strains is responsible for their adhesion to CEF cells. This recombinant antigen can be used in adherence study and as a candidate recombinant immunogen for the protection of chicken from fowl cholera.

References


Molecular characterization of adhesive protein of avian *P. multocida*

**Pasteurella multocida** P-1059 株及び変異 P-1059B 株の付着性蛋白の分子解析

Entomack Borrathybay*1*・Nattawooti Sthitmatee*1**・鈴木健一*1*・新中須亮*1*・土田修一*2*・阿久澤良造*3*・片岡康*1*・澤田拓士*1*

*1* 日本獣医生命科学大学獣医学部家畜微生物学教室
*2* 日本獣医生命科学大学獣医学部比較細胞生物学教室
*3* 日本獣医生命科学大学応用生命科学部乳肉利用学教室

*現所属：中国 高等大学 生物資源環境科学院
**現所属：タイ チェンマイ大学 獣医学部
