**Inhibition of Capsular Protein Synthesis of *Pasteurella multocida* Strain P-1059**

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**ABSTRACT.** A mutant strain, PBA322, was constructed by electroporation of a phagemid containing the coding region of antisense RNA of the *ompH* gene, encoding 39 kDa capsular protein or OmpH, into the parental strain P-1059 (serovar A:3) of *Pasteurella multocida*, and the pathogenicity was determined in mice and chickens. Grayish colonies of the mutant, indicating loss of capsule synthesis, were observed under a stereomicroscope using obliquely transmitted light, while iridescent colonies were observed for the parental strain. Moreover, strain PBA322 showed a low amount of OmpH compared with the parental strain on SDS-PAGE. Additionally, the capsule of strain PBA322 was thinner than that of the parental strain according to electron microscopy, correlating to the attenuation against chickens. In conclusion, strain PBA322, the mutant of *P. multocida* strain P-1059, was completely attenuated for chickens.

**KEY WORDS:** antisense RNA, fowl cholera, noncapsulated strain, *Pasteurella multocida*.

*Pasteurella multocida*, a gram negative, capsulated coccobiacillus, is the causative agent of fowl cholera, bovine and buffalo hemorrhagic septicaemia and swine atrophic rhinitis. Capsular serogroup A and somatic serotypes 1, 3 and 4 are the predominant forms in fowl cholera [13, 22–25]. Observation of a colony under a stereomicroscope using obliquely transmitted light can be used to differentiate *P. multocida* capsulated (iridescent) and noncapsulated (blue or gray) strains [23]. Our previous studies demonstrated that the 39 kDa protein of strain P-1059 (serovar A:3), which is the OmpH protein, was correlated with the colonial appearance, capsule thickness, adherence ability and pathogenicity for chickens [1–4,12]. The noncapsulated variant strain P-1059B spontaneously developed from *P. multocida* capsulated strain P-1059 demonstrated loss of ability to produce OmpH, resulting in a marked loss of virulence [3]. This indicated that OmpH was correlated with the pathogenicity of the bacteria for chickens. In the present paper, we describe the construction of an attenuated mutant of *P. multocida* strain P-1059 by electroporation of phagemid-contained coding region of the antisense RNA of the *ompH* gene into parental strain P-1059. Additionally, its pathogenicity for chickens was determined.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media and growth conditions:** The bacterial strains and plasmids used in this study are listed in Table 1. *P. multocida* strains were cultured in tryptose broth (TB; Becton, Dickinson and Company, Sparks, MD, U.S.A.) at 37°C for 6 hr and were then cultured on dextrose starch agar (DSA; Becton, Dickinson and Company) at 37°C for 18 hr. Colonies of *P. multocida* strains was used to determine the biochemical characteristics, urea, indole, fermentations of mannitol, glucose, sucrose, galactose, xylose, mannose and lactose, and incubated at 37°C for 18 hr. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) broth or on LB agar plates supplemented with 100 µg/ml ampicillin (Sigma-Aldrich, St. Louis, MO, U.S.A.), 20 mM IPTG (Takara, Shiga, Japan) and 80 µg/ml X-Gal (Takara). Plasmids were used for cloning and electroporation as described in the manufacturer’s instructions.

**Construction of antisense RNA of the ompH gene:** The coding region of antisense RNA of the *ompH* gene of strain P-1059 was digested with *Bam*HI and *Bgl*II from *P. multocida* strain P-1059 and ligated into the vector, pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.), 20 mM IPTG (Takara, Shiga, Japan) and 80 µg/ml X-Gal (Takara). Plasmids were used for cloning and electroporation as described in the manufacturer’s instructions.

**Sequencing:** Sequence determinations were performed using M13 forward and M13 reverse primers (Takara) with BigDye® Terminator cycle sequencing kit (AB Applied Biosystems, Foster City, CA, U.S.A.) and generated with an ABI Prism® 310 Genetic Analyzer (AB Applied Biosystems). Sequencing analysis was conducted with Applied Biosystems DNA Sequencing Analysis Software Version 5.1 (AB Applied Biosystems).
Electroporation: 

*P. multocida* strain P-1059 was cultured in TB broth at 37°C until the OD_{600} reached 0.5–0.7 (mid-log phase). Bacterial cells were pelleted by centrifugation at 15,000 × g for 15 min and then washed twice with 10% sterile glycerol. Finally, the cells were resuspended with 10% sterile glycerol and kept on ice.

Electroporation was performed as described previously [14] with the Gene Pulser™ apparatus (Bio-Rad, Foster City, CA, U.S.A.) set at 2.5 kV, 25 μF and 600 Ω (field strength 25 kV/cm). Following electroporation, the cell suspensions were cultured in TB broth without selection at 37°C for 2 hr and then cultured on selective Colombia blood agar (Oxoid, Hampshire, UK) supplemented with ampicillin (100 μg/ml) at 37°C for 18 hr. Then, colonies were selected and subcultured on selective DSA plates supplemented with 100 μg/ml ampicillin. Colonies on DSA plates were observed under a stereomicroscope using obliquely transmitted light. The noniridescent colonies were selected and cultured in TB broth at 37°C for 6 hr before being kept in 10% sterile glycerol at −80°C until use.

Crude capsular extract (CCE): CCEs of *P. multocida* strains were prepared using the saline extraction method as described previously [27]. CCEs were kept at −20°C until use.

Electron microscopy: Bacterial cells were prepared for transmission electron microscopy as described previously [3, 17]. Briefly, frozen stock cultures of *P. multocida* strains were inoculated into fresh TB medium and incubated at 37°C for 6 hr, and then 0.25 ml of the broth culture was plated on DSA plates and incubated at 37°C for 18 hr. The bacteria grown on the DSA 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 hr, and the bacteria were collected by centrifugation at 4800 × g for 10 min. The pelleted bacterial cells were suspended in 0.05 M cacodylate buffer and allowed to react with the polycationic ferritin (final concentration, 1.0 mg/ml, 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% agar (Aga Noble; Becton, Dickinson and Company), washed three times in cacodylate buffer and postfixed with 2% osmium tetroxide for 1 hr. The specimens were dehydrated in graded ethanol and embedded in an epoxy resin mixture. Thin sections of the embedded specimens were stained with uranyl acetate and lead citrate and then observed by electron microscope (Hitachi, H-7100 type, Tokyo, Japan) at an acceleration voltage of 75 kV at calibrated magnification. Capsule thickness was recorded by measuring the lengths at four sites of the capsule for the long and short axes of each cell. Averages of the capsule thickness from 100 cells of each strain were used for statistical analysis by Student’s t-test.

Animal: Eight-week-old *P. multocida*-antibody-free layers (Hi-sex, RPM Farm & Feed Co., Ltd., Chiang Mai, Thailand) were used in this study. The Animal Use and Care Committee of Chiang Mai University ensured that use of the laboratory animals complied with the guidelines for laboratory animal ethics. Experiments were performed in a closed-system building under a biosafety system to prevent spread of bacteria into the environment. The experiment rooms and equipments used in this study were cleaned with a disinfectant (sodium hypochlorite) 2 weeks before and after experiments. Waste products were also treated with the disinfectant before released into the environment.

Pathogenicity test: Experiments are described in Table 3. Briefly, chickens were intravenously or intramuscularly inoculated with a serial tenfold dilution of the mutant. In addition, chickens were subcutaneously inoculated with 0.5 ml of strain P-1059 bacterin as the positive control vaccine. Clinical signs were observed for 10 days post inoculation. Necropsies were performed in order to observe the lesions of dead birds.

**SDS-PAGE and Western blotting:** Proteins were analyzed
on a 12.5% polyacrylamide slab gel according to Laemmli’s method [18] in a mini-slab apparatus (ATTO Corporation, Tokyo, Japan) and stained with Coomassie blue R-250 (Sigma) [19]. Separated proteins were transferred to the nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) by a semi-dry system (ATTO Corporation) and immunostained with chicken anti-OmpH sera at a dilution of 1:100 and with 1:1,000 dilutions of horseradish peroxidase-conjugated anti-chicken IgY (IgG; Sigma). Lastly, transferred proteins were visualized using 3,3-diaminobenzidine (DAB; 20 mg/ml, Sigma) in phosphate-buffered saline (PBS, pH 7.2) as a chromogenic substance.

RESULTS

Construction of mutants: The digested DNA cassette of theompH gene was approximately 1.1 kbp in size on 1.5% agarose gel (Fig. 1). After purification, DNA were A-tail added prior to ligate into pGEM-T Easy vector, white colonies were selected and one clone with the exact sequence was chosen to designate E. coli strain PGECP391. Plasmid pGECP391 was prepared from E. coli strain PGECP391 and then digested withBamHIandXhoI to obtain the ompH gene cassettes. These cassettes were approximately 1.1 kbp in size on 1.5% agarose gel (Fig. 1). Purified ompH gene cassettes were ligated into predigested pBluescript II SK at the same restriction site, white colonies were selected and one clone with the exact desired sequence of the ompH gene was chosen to designate E. coli strain PBCP391. Plasmid pBCP391 was prepared and introduced into wild-type electrocompetent cells by electroporation, while electroporation with or without pBluescript II SK were performed as a control. Approximately 100 colonies of cells with phagemids were observed on selective Colombia agars, whereas no growth was observed in cells without phagemid plates. Colonies were selected and subcultured on selective DSA. Then, the colonies were observed under a stereomicroscope using obliquely transmitted light after incubation at 37°C for 18 hr in order to observe the colonial color.

Characterization of colonies: Thirty colonies were selected for determination of the appearance and biochemical characteristics of colonies. Approximately 30 grayish colonies of cells with an ompH cassette-inserted phagemid were observed, whereas the cells without insertion of a phagemid were still iridescent (Fig. 2). The biochemical characteristics of the colonies are shown in Table 2. As observed, the mutant showed no difference in biochemical characteristics compared with the parental strain.

Transmission electron microscopy: The cell morphology of P. multocida strains before and after inhibition of capsular protein was observed by transmission electron microscopy (Fig. 3). Parental strain P-1059 possessed a thick and regular capsule with an average thickness of 101.2 ± 35.9 nm (Fig. 3, left). In contrast, strain PBA322 possessed a thin capsule with an average thicknesses of 25.5 ± 1.6 nm (Fig. 3, right). Moreover, there was significant difference in capsule thicknesses between these two strains.

Attenuation of virulence in chickens: Strain PBA322 also showed no pathogenicity in chickens in comparison with...
wild-type strain inoculations, even intramuscular or intravenous inoculation with $10^8$ cfu of the bacteria (Table 3). Chickens started clinical signs such as depression and anorexia, at 6 to 8 hr after inoculation with the wild-type strain. Then, chickens started to die at 12 hr after inoculation, and dead chickens were found until 3 days post inoculation. The carcasses showed the typical gross lesions of fowl cholera, e.g., multiple necrotic foci at the liver and/or spleen, lung congestion and edema, multiple petechiae in the liver, hemorrhage in the small intestines and splenomegaly. Bacterial isolation showed pure colonies on the agar plates, and the biochemical reactions of the isolates showed the typical properties of *P. multocida* species (data not shown).

**SDS-PAGE and immunoblotting:** Whole-cell lysates of strains P-1059 and PBA322 and purified recombinant OmpH (rOmpH protein) [26] of strain P-1059 were analyzed on SDS-PAGE slab gel. Strain PBA322 showed small amounts of OmpH on SDS-PAGE when compared to the parental strain P-1059 (Fig. 4). Moreover, whole-cell lysates of strain P-1059 and purified rOmpH protein were immunostained with the chicken antisera against OmpH, whereas whole-cell lysate of strain PBA322 was not (Fig. 4).
DISCUSSION

Mutants of *P. multocida* have also been constructed by molecular techniques [7, 8, 11, 15, 28]. These mutants were attenuated and could induce effective protection against *P. multocida* infection in vivo. Inhibition of gene expression by antisense RNA is the method used to control or inhibit gene expression in many organisms including bacteria [20, 29]. A gene encoding antisense RNA can be introduced easily into organisms using plasmid vectors, and the antisense

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Table 3. Test of pathogenicity conferred by strain PBA322 for chickens

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of chickens (IV/IM)</th>
<th>Inoculum dose (cfu per 0.2 ml)</th>
<th>Survivors % (IV / IM)</th>
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<tr>
<td>Control</td>
<td>5 / 5</td>
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<td>100</td>
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<tr>
<td>P-1059</td>
<td>5 / 5  2.5 × 10^8</td>
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<td>5 / 5  2.5 × 10^7</td>
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<td>5 / 5  2.5 × 10</td>
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<tr>
<td>PBA322</td>
<td>5 / 5  3.5 × 10^8</td>
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a) IV / IM: Intravenous or intramuscular inoculations in chickens.
b) Dose of intravenous or intramuscular inoculations.
c) Percentage of survivors after inoculations.

Fig. 4. SDS-PAGE (a) and immunoblotting (b) of *P. multocida* strains P-1059, PBA322 whole-cell lysate and purified recombinant OmpH protein. Lanes: M, molecular mass standards; 1, whole-cell lysate of strain P-1059; 2, whole-cell lysate of strain PBA322; and 3, purified recombinant OmpH protein.
RNA will be transcribed and inhibit protein synthesis by binding to messenger RNA (mRNA) of the target protein [9]. The advantage of this method is that it is an easy way to construct a knockout organism or study control of the expression of an interesting gene.

Colonies of capsulated and noncapsulated strains of *P. multocida* are easily compared on transparent agar media by observation under a stereomicroscope using obliquely transmitted light [23]. Colonies of capsulated strains appear yellowish-green, bluish-green or pearl-like iridescent, whereas colonies of noncapsulated strains are small and not iridescent, but instead appear blue, grayish-blue or gray. Dissociation, which is a manifested form, is associated with loss of the bacterial capsule. In addition, dissociation of colonies from iridescent to noniridescent and the concomitant loss of the capsule in virulent strains are associated with the reduction or loss of virulence. Noncapsulated strains have often been from chronic cases and show low virulence but are still pathogenic to the natural hosts [16, 25]. However, a noncapsulated strain (P-1059B) could be obtained from 35 serial passages of *P. multocida* strain P-1059 [3]. This strain produced a low amount of OmpH and seemed to be avirulent for chickens [3]. Therefore, inhibition of OmpH synthesis was thought to reduce the pathogenicity of *P. multocida* in the host. Strain PBA322 in the present study was constructed by inhibiting expression of OmpH with the antisense RNA of the ompH gene. The antisense RNA of the ompH gene was successfully transcribed in vitro. As a result, strain PBA322 produced a gray colony on DSA plates compared with the capsulated strain when observed under a stereomicroscope using obliquely transmitted light (Fig. 2). The gray colony, indicating the thin capsules, indicated that the antisense-ompH RNA was transcribed in vivo and remarkably inhibited capsular protein synthesis.

The amounts of OmpH were correlated with capsule thickness and the bacterial pathogenesis [3]. Strains with low amounts of OmpH or that were noncapsulated lost the ability to adhere to CEF cells compared with a capsulated strain. Interestingly, the low virulence strain showed low amounts of OmpH on SDS-PAGE. This demonstrated that the OmpH were correlated with the presence of a bacterial capsule and adhesion to host cells. Additionally, previous studies suggested that reduction of the amounts of capsule or acapsular variants of *P. multocida* capsular serogroup A strains caused bacteria to lose the ability to adhere to turkey air sac macrophages or alveolar macrophages [6, 10, 21]. Similarly, SDS-PAGE in the present study showed that strain PBA322 had a low amount of OmpH. Moreover, immunoblot analysis showed that whole cells of strain PBA322 were immunostained slightly with the chicken antiserum against native OmpH compared with the parental strain and purified OmpH (Fig. 4). These results indicated that strain PBA322 lost the ability to synthesize OmpH, and this might affect to the pathogenicity for chickens (Table 3).

In conclusion, we successfully constructed an antisense RNA of the OmpH gene-based mutant strain PBA322 from *P. multocida* strain P-1059. Grayish colonies on DSA plate observed under a stereomicroscope using obliquely transmitted light indicated that strain PBA322 lost the ability to synthesize capsule. Moreover, strain PBA322 was an attenuated strain for chickens. Therefore, a noncapsulated strain of *P. multocida* strain P-1059 was constructed by inhibition of OmpH synthesis using the antisense RNA method.

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REFERENCES