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Characterization of a lipopolysaccharide-protein complex of type A *Pasteurella multocida*

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Pasteurella multocida type A의 lipopolysaccharide-protein 복합체의 특성

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Abstract : An immunogenic, high molecular weight lipopolysaccharide (LPS)-protein complex isolated from a potassium thiocyanate extract of a *Pasteurella multocida* (*P multocida* ; strain P-2383, capsular type A and somatic type 3) was characterized. Chemical analysis of the complex by gas chromatography on a capillary column demonstrated that this complex contained most of the chemical constituents characteristic of LPS extracted by the phenol-water method from the whole bacterium. However, there was proportionately more carbohydrate than fatty acid in the complex in contrast to LPS in which fatty acid seemed to be in excess. When toxicity of the complex was evaluated in 10-day-old chicken embryos, the complex was less toxic (LD_{50} = 12.72μg) than the purified LPS (LD_{50} = 0.44μg). The LD_{50} of the LPS moiety extracted from the complex was 5.24μg. Composition of the complex was analyzed by SDS-PAGE with silver staining and Western immunoblotting. The complex did not migrate through the polyacrylamide gel unless dissociated with SDS. The complex dissociated with SDS contained at least 32 different protein and polysaccharide components: 18 components reacted with an antiserum against the complex. There was no significant compositional variation between the complexes from different strains, but quantitative differences in individual components were noted. When cross-protectivity of the complex was evaluated in mice, this complex provided substantial protection not only against the homologous bacterium but also against different *P. multocida* strains of the same serotype. LPS-protein complexes isolated by the same method from other

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strains also induced protection against an challenge with P-2383.

Key words : lipopolysaccharide-protein complex, *Pasteurella multocida* , protection.

Introduction

Capsular type A and somatic type 3 strains of *Pasteurella multocida* (*P multocida*) are important etiologic agents in fowl cholera, pneumonic pasteurellosis of cattle and sheep, and a variety of clinical syndromes in rabbits[1-4]. *Pasteurella* bacterins have been utilized as immunoprophylactics in cattle for many years but usage is now limited due to detrimental effects and questionable efficacy[5-9]. Therefore, attenuated organisms[10,11] have been evaluated, and demon-strated to be immunogenic as well as certain disadvantages[4]. More recently, extensive experiments have been conducted on the immunogenicity of subcellular fractions of the microorganism[4,12-26]. Lipopolysaccharides(LPSs) present in the outer membrane of many Gram-negative bacteria are known to play important roles in bacterial pathogenesis since they exhibit many endotoxic activities in a host such as pyrogenicity, depression, diarrhea, and lethality for chicken embryos, mice, rabbits and chickens[27-31]. LPS extracted from *P multocida* has also been reported to evoke such endotoxin activities[15,32,33] Also, many researchers have suggested that the *P multocida* fractions which possessed immunogenicity contained LPS as an important constituent[12-17,23,25]. The presence of LPS in immunogenic fractions of *P multocida* has been determined by serologic cross-reactivity of the fractions with the purified LPS[15,16,23,25], endotoxin activities in experimental animals and chicken embryos[12,13,15-17], or detection of 2-keto-3-hydroxyl-octonate(KDO) and/or fatty acid by chemical methods [12,13,17,23]. While these determinations may have suggested the presence of LPS, they failed to relate basic chemical differences between the immunogenic fractions and purified LPS. Previously, we have reported the isolation of an immunogenic fraction from a potassium thiocyanate(KSCN) extract of *P multocida* (strain P-2383 ; capsular type A and somatic type 3) by sucrose-density gradient centrifugation[25]. This fraction, called P-2383-1, was highly immunogenic in mice and contained 27% protein and 12% carbohydrate. Although the fraction crossreacted serologically with LPS extracted from the whole bacterium by Westphal's phemol-water procedure, KDO was not detected in P-2383-1 at a protein concentration of 5mg/ml. The objectives of this study were to determine the endotoxin LPS content of P-2383-1 and another LPS-containing immunogen of *P multocida* by gas chromatography on a fused-capillary column, to evaluate P-2383-1 for its toxic activity in chicken embryos, and to discern immunologic relationships between the LPS-protein complexes from different strains of the serotype through compositional analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting.

Materials and Methods

Organism and preparation of LPS-protein complexes : Five *P multocida* strains isolated from pneumonic bovine lungs submitted to the Iowa Veterinary Diagnostic Laboratory of Iowa State University were used in this experiment. These isolates were provided a designation (P-2383, P-9238, P-9663, P-9954 and P-10027) and determined to be capsular type A and somatic type 3. Culture conditions for growth of *P multocida* organisms and preparation of KSCN extracts have been described previously[25] An immunogenic LPS-protein complex, called P-2383-1, was isolated from a KSCN extract of an encapsulated *P multocida* (P-2383) by sucrose-density gradient centrifugation[25]. LPS-protein complexes equivalent to P-2383-1, were also prepared from the other strains by the same method and named P-9238-1, P-9663-1, P-9954-1 and P-10027-1. Another immunogenic LPS-protein complex, called P-1059-40p, was isolated from a 0.85% NaCl extract of a nonencapsulated variant (P-1059 ; capsular type A and

somatic type 3) by gel-filtration as described by Ganfield *et al*[17].

Fractionation of the P-2383-1 complex : P-2383-1 was fractionated into LPS and protein moieties by an extended Wesphal's phenol-water procedure described by Sultzer and Goodman[34]. Briefly, P-2383-1 dissolved in 0.32M NaCl/0.01M Tris-HCl buffer was dialyzed against distilled water at 4℃ for 2 days and warmed to 65℃ before treatment with an equal volume of 90% phenol (65℃). The LPS moiety (P-2383-1-LPS) was isolated from the water phase while the protein moiety (P-2382-1-PRO) was collected from the phenol phase by precipitation with cold ethanol (-20℃). Since P-2383-1-PRO was poorly soluble in water, it was solublized by suspending 2mg (dry weight) in 0.95ml of distilled water and the addition of 0.05ml of 0.1N NaOH.

Preparation of *P multocida* LPS : Phenol-water extracted LPS(PW-LPS) was prepared from strain P-2383 by the method of Westphal and Jann[35] with slight modifications as described previously[25].

Chemical analysis : Bacterial fractions were assayed for basic chemical content. Total carbohydrate was determined by the phenol-sulfuric acid procedure[36] using glucose as a standard. Protein content was determined calorimetrically from the reaction of protein with Serva blue G dye (Serva Fine Chemicals, USA) using bovine serum albumin (Sigma, USA) as a standard[37].

Gas chromatography(GC) : Derivatization of samples for GC analysis was performed by the method of Bryn and Jantzen 38 with slight modifications. Briefly, 2 to 5mg of the sample was suspended in one ml of 2M HCl in a teflon-lined screw-capped vial and held at 85℃ for 18h. The methanolysates was concentrated to dryness at room temperature with nitrogen gas and trifluoroacetylated by adding 0.2ml of 50% trifluoroacetic acid (Aldrich USA) in acetonitrile (Aldrich) and heating in a boiling water bath for 2 min. After cooling to room temperature, the reaction of 10% was injected into the column. Galactose, glucose, KDO and LD-heptose (Sigma) were used for carbohydrate standards and a bacterial fatty acid mixture was used as a fatty acid standard. The GC analysis was carried out on a Hewlett-Packard 402 GC system that had been modified for the use of a capillary column and equipped with a flame-ionization detector. A fused-silica capillary column (Foxboro/Analabs, USA) was operated in a spilt mode and with helium carrier gas. The column temperature was held for 5 min at 95℃ after injection of the sample and then programmed to increase at 4℃/min up to 230℃.

Production of rabbit antiserum : Procedures for production of rabbit antiserum to *Pasteurella* fractions have been described previously[25]. White New Zealand female rabbits, immunized with P-2383-1, provided antiserums that produced a single precipitation line in crossed-immunoelectrophoresis with the immunizing antigen and two precipitation lines hen reacted with whole KSCN extract[25].

Chicken embryo lethality : The chicken embryo lethality test was conducted by the method of Smith and Thomas[39]. Groups of five 10-day-old embryonated eggs were inoculated on the chorioallantoic membrane with 0.1ml volumes of 2-fold dilutions of the *P multocida* fractions. The eggs were incubated at 37℃ and candled for viability at 24h and 48h. The fifty percent lethal dose (LD_{50}) was determined by the method of Reed and Muench[40].

SDS-PAGE : Discontinuous SDS-PAGE was performed with 10% separating gel and 5% stacking gel by the method of Laemmli[41] with slight modifications. Sample preparation buffer (100ml) contained 2g of SDS, 10ml of glycerol, 10ml of 0.04% bromophenol blue, 12.5ml of 0.5M Tris-HCl (pH 6.8), and 67.5ml of distilled water. Immediately prior to use for treatment of samples, 0.1ml of 2-mercaptoethanol was added to 10ml of the buffer. One-tenth ml of this solution was added to the same volume of an individual sample and the mixture was heated for 4 min in a boiling water bath (95℃). One-tenth mg of proteinase K (PK) solubilized in 10μl of the sample preparation buffer was added to the sample mixture subject to protein digestion and incubated for 1h at 60℃. Samples were loaded onto the polyacrylamide gels in a volume of 20 to 30μl and separated for 6 to 7h at 25mA per gel (16cm × 11.5cm × 0.15cm). After electrophoresis, the gel was fixed overnight in 25%(vol/vol) 2-propanol- 0%(vol/vol) acetic acid solution. Silver staining was conducted with a silver staining kit (Bio-Rad) according to the recommended procedures.

Western immunoblotting : Western immunoblotting was performed according to the method of Towbin *et al*[42]. The

polyacrylamide gel subject to immunoblot was washed for 1 h in the blotting buffer solution containing 25mM Tris-HCl, 192mM glycine and 20% (vol/vol) methanol (pH 8.3). Separated components in the gel were electroblotted to a nitrocellulose membrane (Bio-Rad) for 5h at 21mA. The membrane was incubated overnight in a Tris-buffered saline solution (TBS ; 20mM Tris-HCl, 0.5M NaCl, pH 7.5) containing 3% bovine serum albumin. The membrane was washed three times in TBS containing 0.0005% (vol/vol) of Tween 20 (TTBS) and then incubated overnight in rabbit antiserum against P-2383-1 diluted 1 : 100 in TTBS containing 1% gelatin. The membrane was washed 3 times with TTBS and incubated for 1 h in a TTBS-1% gelatin containing solution 1 : 500 peroxidase-labelled goat anti-rabbit IgG (Sigma). The membrane was washed twice with TTBS and once with TBS, and incubated in TBS containing 60mg HRP color development reagent (Bio-Rad) and 60μl of 20% (vol/vol) hydrogen peroxide.

Mouse immunization and challenge : White Swiss mice (Bio-Lab Corp, USA) of one sex, 8 weeks of age, and at least 18g in weight were immunized subcutaneously with 0.1 ml (1mg/ml protein concentration) of antigen and challenged intraperitoneally 2 weeks later with 0.1ml of bacterial suspension (100 to 200CFU). Mice were observed for 1 week after the challenge and deaths recorded.

Results

Chemical analysis : The LPS-protein complexes of several *P multocida* strains, equivalent to P-2383-1, contained 5 to 27% carbohydrate and 22 to 38% protein. The LPS moiety extracted from P-2383-1 contained 50% carbohydrate and 7% protein while P-2383-1-PRO contained less than 2% carbohydrate and 100% protein.

GC analysis : GC analysis indicated that the constituents of PW-LPS of *P multocida* were galactose, glucose, LD-heptose, glucosamine, KDO, 3-hydroxyl-dodecanoate, tetradecanoate, 3-hydroxy-tetradecanoate and hexadecanoate (Fig 1). As illustrated in Fig 2, both LPS containing immunogens, P-1059-40p and P-2383-1, contained most of chemical constituents present in PW-LPS of *P multocida* except that KDO was difficult to detect in P-2383-1. However, differences were observed in GC patterns of the three preparations indicating quantitative variations within the shared constituents. P-2383-1 contained a higher ratio of carbohydrate to fatty acid than P-1059-40p and PW-LPS. The immunogenic fractions contained considerable amounts of long chain fatty acid such as hexadecanoate that were present in limited quantity in PW-LPS.

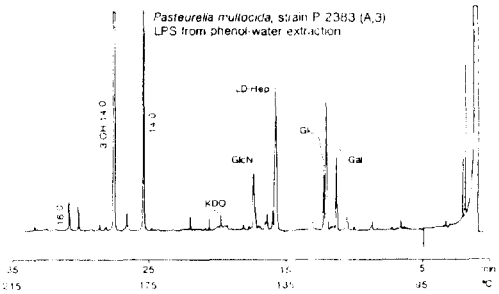

Fig 1. Gas chromatogram of phenol-water extracted LPS of *P multocida* (strain P-2383) after methanolysis and trifluoroacetylation. Conditions of sample preparation and chromatography are given in the text. Abbreviations : Gal, galactose ; KDO, 2-keto-3-hydroxy-octonate ; 3-OH-12:0, 3-hydroxy-dodecanoate; 14:0, tetradecanoate; 3-OH-14:0, 3-hydroxytetradecanoate; 16:0, hexadecanoate.

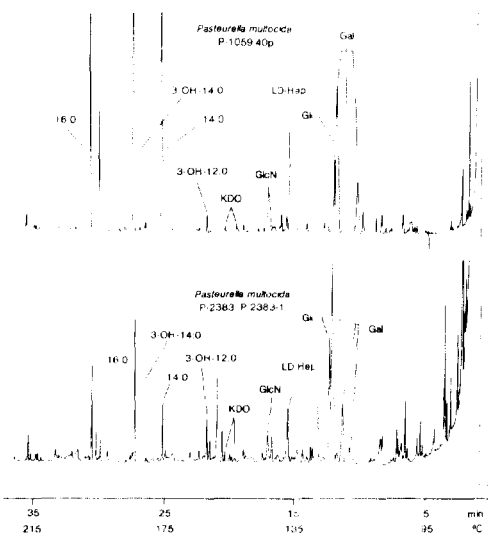

Fig 2. Gas chromatogram of LPS-containing immunogens of *P multocida*.
Abbreviations are listed in the legend of Fig 1.

Chicken embryo lethality : Results of toxicity assays, its

Table 1. Lethality for chicken embryos of fractions extracted from *P multocida*

Dosage(μg)	PW-LPS	P-2383-1	P-2382-1-LPS
64	---[a]	6/6[b]	---
32	---	4/6	---
16	---	4/6	6/6
8	---	3/6	5/6
4	6/6	0/6	2/6
2	6/6	0/6	1/6
1	5/6	---	0/6
0.5	4/6	---	---
0.25	1/6	---	---
0.125	0/6	---	---

[a] Not done.
[b] No. of dead/no. tested.

LPS moiety (P-2383-1-LPS) and PW-LPS in 10-day old chicken embyros are listed in Table 1. PW-LPS exhibited the highest toxicity with a LD_{50} of 0.44μg while P-2383-1 exhibited the lowest toxicity with a LD_{50} of 12.72μg. The LD_{50} of the LPS moiety extracted from P-2383-1 was 5.24μg.

Compositional analysis of the LPS-protein complexes : The LPS-protein complexes that were not treated with SDS failed to migrated through the polyacrylamide gel. The complexes treated with SDS showed visible bands in the polyacrylamide gel at a 0.5mg/ml protein concentration ; however, the purified protein moiety of P-2383-1 required at least 4mg/ml protein concentration to show visible bands. The LPS-protein complexes contained at least 3? protein and polysaccharide components with the molecular size ranging from less than 14kDa to greater than 94kDa (Fig 3). Most of these components were protein in nature since they were destroyed by proteinase K treatment (Fig 4). At least 4 poly-

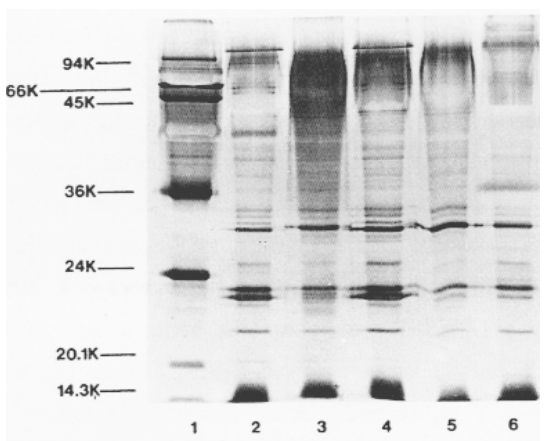

Fig 3. SDS-PAGE profiles of LPS-protein complexes isolated from *P multocida* strains of capsular type A and somatic type 3 as revealed by silver staining. The lanes and fractions were as follows: 1, molecular weight standard; 2, P-2383; 3, P-9238-1; 4, P-9663-1; 5, P-9954-1; 6, P-10027-1.

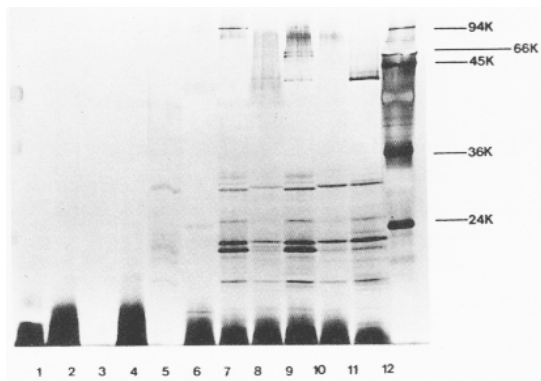

Fig 4. SDS-PAGE profiles of the fractions of *P multocida* as revealed by silver staining. Some fractions were treated with proteinase K(PK). The lanes and fractions were as follows: 1, PW-LPS; 2, P2383-1-LPS+PK 3, P-2383-1-PRO+PK 4, P-2383-1-LPS; 5, P-2383-1-PRO 6, P-2383-1+PK; 7, P-2383-1; 8, P-238-1; 9, P-9663-1; 10, P-9954-1; 11, P-10027-1; 12, molecular weight standard.

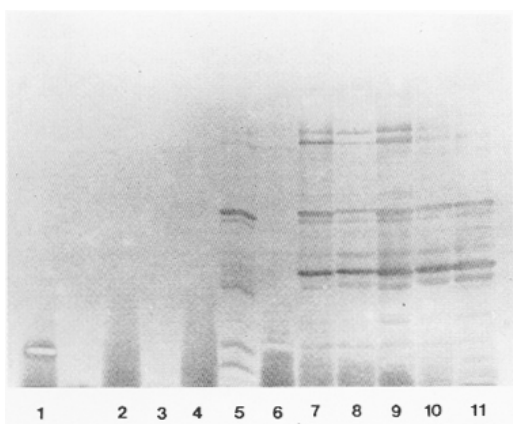

Fig 5. Western immunoblot of the fractions of *P multocida* stained by peroxidase-labeled goat anti-rabbit IgG. Some fractions were treated with protein kinase(PK). The lanes and fractions were as follows: 1, PW-LPS; 2, P2383-1-LPS+PK 3, P-2383-1-PRO+PK 4, P-2383-1-LPS; 5, P-2383-1-PRO 6, P-2383-1+PK; 7, P-2383-1; 8, P-238-1; 9, P-9663-1; 10, P-9954-1; 11, P-10027-1.

saccharide bands of different molecular sizes were identified; however, the predominant band was of smaller molecular size (Fig 4). Most of these components were shared between the complexes isolated from different strains, but the quantity of individual components was somewhat variable. Analysis by Western immunoblotting (Fig 5) indicated that at least 18 of those components reacted with an antiserum against P-2383-1.

Immunogenecity in mice : As indicated in Table 2, groups of mice immunized with the LPS-protein complexes were protected against a challenge infection with strain P-2383. Mice immunized with P-2383-1 were *P multocida* strains that killed 100% of the control mice (Table 3).

Table 2. Immunogenicity for mice of LPS-protein complexes of *P multocida* against challenge exposure with strain P-2383. Each mouse received 100 to 200 CFU of the bacterium intraperitoneally

Immunization	No. surviving/no. tested
None	0/5
P-9238-1	4/5
P-9663-1	5/5
P-9954-1	5/5
P-10027-1	5/5

Table 3. Immunogenecity for mice of LPS-protein complex, P-2383-1, against challenge infections with heterologous *P multocida* strains of capsular type A and somatic type 3

	Immunization	
Challenge strain	Control	P-2383-1
P-9238	0/5[a]	5/5
P-9663	0/5	5/5
P-9954	0/5	5/5
P-10027	0/5	5/5

[a] No. surviving/no. tested.

Discussion

We have previously reported that a LPS-protein complex (P-2383-1) isolated from a KSCN extract of a *P multocida* strain (P-2383) induced resistance in mice to challenge infection with the homologous strain[7,8]. P-2383-1 serologically cross-reacted with PW-LPS of the organism, but KDO was not detected in P-2383-1 at a protein concentration of 5mg/ml. Previous experiments by other workers have demonstrated the presence of LPS in immunogenic *P multocida* fractions as determined by serological assays[15,16,23,25], endotoxin activity[12,13,15-17], and chemical methods[12,13,17-23]. However, those determinations were quite restrictive in relating LPS as a component of the respective fractions. Serologic reactions involving polysaccharide antigens are widely recognized for cross-reactivity[27,28]. Therefore, cross-reaction between PW-LPS and immunogenic fractions does not necessarily mean the presence of LPS but does not rule out potential toxic materials other than LPS present in cell wall materials of bacteria[11]. Fatty acids are associated with LPS but are common constituents of the bacterial membrane structure[11]. Detection of KDO in *P multocida* fractions has been used most extensively to determine the presence of LPS; however, Rimler *et al*[23] reported that the amount of KDO in LPS of somatic serotype 3 *P multocida* was only approximately 1%. Therefore, detection of KDO in an immunogenic fraction of *P multocida* may be difficult if LPS represents only a minor component of the fraction. GC analysis of the *P multocida* fractions in this study indicated the presence of LPS in the two immunogenic fractions (Fig 2) since typical constituents of the bacterial LPS was demonstrated. Also, these chromatograms clearly showed compositional differences between the two fractions. This was particularly interesting since previous studies indicated that they contained the same protein and carbohydrate content[17,25]. The differences in ratio of polysaccharide to fatty acid between the immunogenic fractions and PW-LPS may be explained by the unique nature of the cell wall structure of Gram-negative bacteria. The cell wall is a multilayered structure including a cytoplasmic membrane, a thin rigid layer of peptidoglycan and the trilaminar outer membrane[27,29]. LPS exists in the outer membrane in releasable and nonreleasable forms based on the activity of ethylenediaminetetraacetic acid (EDTA) on bacterial cells. Approximately 50% of the LPS molecules are bound

to each other through divalent cations and forms blebs that can be easily released from the membrane in exponentially growing bacteria or by EDTA treatment. The second fraction of LPS molecules are bound to other molecules in the membrane through hydrophobic and ionic interactions. Therefore, it is possible that different extraction methods used for the preparation of the bacterial fractions may influence the chemical composition of LPS in the products. A chaotrophic reagent, KSCN, may extract a part of LPS present in the outer membrane such as LPS blebs or cleave off some fatty acids from lipid A, while phenol-water treatment may extract the whole LPS and even the inner leaflet of the outer membrane. It is also possible that the purification process utilized in the preparation of the bacterial fractions may have influenced the chemical composition of the final products. The PW-LPS was prepared from bacteria washed several times by centrifugation and could have resulted in a loss of surface materials. P-2383-1 was isolate from unwashed bacteria and should have resulted in retention of most of the cell surface materials. While P-2383-1 obviously contains LPS, there is in addition an abundance of proteins and other polysaccharides. Therefore, the relative amount on LPS in these two preparations was distinctly different. The differing toxicity of the various *P multocida* fractions probably reflects the content and composition of the endotoxin lipid A. The fatty acid content and composition of this lipid moiety is known to influence toxicity of LPS[28,45]. P-2332-1-LPS was less toxic than PW-LPS. The LPS moiety of P-2383-1(P-2383-1-LPS) was more toxic than P-2383-1 but less toxic than PW-LPS. These results were expected since only a part of P-2383-1 is LPS and P-2383-1-LPS contained more carbohydrate than fatty acid in comparison with PW-LPS (Fig 1 and 2).

The variability in relative fatty acid content in these respective LPS-containing fractions probably is responsible for differing toxicity. Also, the presence of non-LPS materials such as other phospholipids could potentially influence toxicity. Jnatzen *et al*[45] reported the presence of large quantities of hexadecanoic acid in *P multocida* cells but very limited amounts were present in our purified LPS preparations. P-2383-1 was previously reported to be a single antigenic component of the crude KSCN extract that contained at least 25 different antigenic components as determined by crossed-immunoelectrophoretic techniques[25]. SDS-PAGE and Western immunoblotting analyses were conducted to find a molecular component responsible for induction of immunity in mice. P-2383-1 was previously determined to be a single macromolecule[23] whose large size was indicated by the inability of P-2383-1 to migrate through polyacrylamide gel. However, silver staining of *P multocida* fractions separated by SDS-PAGE (Fig 3 and 4) indicated that P-2383-1 appeared to be a molecular complex that was composed of at least 32 different protein and polysaccharide components. Analysis by Western immunoblot (Fig 5) indicated that at least 18 different components from P-2383-1 reacted with an antiserum against P-2383-1 that showed a single precipitation line with P-2383-1 and two lines with the KSCN extract. The protective antigen present in this complex may be difficult to define. While P-2383-1-LPS reacted with the antiserum against P-2383-1, it did not induce immunity that protected mice from challenge with *P multocida* (unpublished observations). Purified LPS may lack immunogenicity, a property that is well recognized[46]. Phenol-water extracted protein from P-2383-1 was also demonstrated to lack immunogenicity (unpublished observations). However, phenol-water treatment may have altered some of the proteins in P-2383-1. This assumption would be supported by the fact that about 8 times greater protein concentration was required for the protein moiety than for the LPS-protein complex to show visible bands in SDS-PAGE and Western immunoblot. Also, a major protein band approximately 23kDa in molecular size apparently was altered since the intensity of the band was quite different between P-2383-1-PRO and the original complex (Fig 5). The presence of this protein in immunogenic fractions from cross-protective strains of *P multocida* may indicate its significance as a protective antigen. The results of the mouse protection study indicated that P-2383-1 induces substantial cross-protection against different strains of *P multocida* of the serotype (Table 3). Also, mice immunized with the LPS-protein complexes isolated from different strains were protected against P-2383 (Table 2). This might be expected since the LPS-protein complexes from

different strains were found to contain common antigenic components when examined by SDS-PAGE and Western immunoblot (Figs 3~5). Therefore, the LPS-protein complex isolated from a KSCN extract of a single strain of *P multocida* can potentially provide cross-protective immunogenicity at least within strains of capsular type A and somatic type 3.

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