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Outer Membrane Protein Profiles of *Haemophilus pleuropneumoniae*

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Outer membrane protein profiles of *Haemophilus pleuropneumoniae* were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cells were disrupted by sonication, and outer membrane-enriched fractions were prepared by differential centrifugation and selective solubilization of the inner membrane with sodium *N*-lauroyl sarcosinate. Colony type, growth medium, time of harvest, and in vitro or in vivo passage had no appreciable effect on the protein profiles of the strains examined. Seven patterns were distinguished among the reference strains of the nine capsular serotypes. These patterns were based on the mobility of the major outer membrane proteins migrating in the 39,000- to 44,000-molecular-weight region of the gel, a 16K to 16.5K protein, and a heat-modifiable 29K protein. Strains of serotypes 1 and 9 had identical outer membrane protein profiles, as did strains of serotypes 2 and 6. The reference strains of the remaining five serotypes each had a distinct pattern. The outer membrane protein profiles of 95 field isolates belonging to serotypes 1, 5, 7, and 9 from swine in the midwestern United States were determined and compared with the reference patterns. The results indicate that the population of *H. pleuropneumoniae* is clonal, with three predominant clones distinguished by both serotype and outer membrane protein profile responsible for the majority of *H. pleuropneumoniae* disease occurring in swine in the United States.

Haemophilus pleuropneumoniae is the etiologic agent of swine pleuropneumonia. The course of the disease can range from peracute to chronic; the characteristic acute lesion is a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (23). Prevalence of this disease has increased dramatically in a large segment of the U.S. swine population in recent years, reflecting in part the associated stresses brought on by intensified production.

Isolates of *H. pleuropneumoniae* have been grouped into eight serotypes based on capsule-associated, heat-stable and heat-labile, type-specific antigens (11, 21, 24, 32; R. Nielsen, Ph.D. thesis, Royal Veterinary and Agricultural University, Copenhagen, Denmark, 1982). The existence of a ninth serotype has recently been proposed (R. Nielsen, Acta Vet. Scand., in press; J. Nicolet, personal communication). A number of studies have focused on the capsule, LPS, hemolysin, and whole-cell proteins of *H. pleuropneumoniae* (18, 22; for review, see reference 33). However, little is known about the outer membrane proteins (OMPs) of the organism. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs of other gram-negative pathogens has resulted in the identification of potentially useful epidemiologic, morphologic, and virulence markers.

The purpose of this study was to examine the SDS-PAGE profiles of outer membrane-enriched fractions of *H. pleuropneumoniae* isolates representing the different capsular serotypes. The influence of solubilization conditions on the OMP profiles of the *H. pleuropneumoniae* serotypes was examined. The genotypic and phenotypic stabilities of the OMP profiles were also determined, as were the effects of growth medium and time of harvest. Lastly, patterns obtained with field isolates from U.S. swine were compared with those of the reference strains to determine the homogeneity of OMPs among strains within the same capsular serotype.

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MATERIALS AND METHODS

Bacterial strains. The sources and designations of the reference strains for *H. pleuropneumoniae* serotypes 1 to 7 were reported previously (31). *H. pleuropneumoniae* 405, serotype 8, and strain CVI 13291, proposed serotype 9, were obtained from H.-J. Riising, Northern Drugs and Chemicals, Ltd., Copenhagen, Denmark. *H. pleuropneumoniae* X1, proposed serotype 9, was obtained from J. Nicolet, University of Bern, Bern, Switzerland. Ninety-five field isolates from different herd outbreaks of pleuropneumonia in Iowa and Illinois were collected and characterized as described previously (31). The isolates were taken from routine submissions to diagnostic laboratories during a 22-month period, from October 1980 until August 1982. Duplicate accessions from the same farm were eliminated. Cultures were maintained by growth in M96 mycoplasma medium (9) and stored at −70°C. Encapsulation was evaluated with a modified India ink negative stain by light microscopic examination (M. D. Graham and R. D. Evans, ASM News, **51**:60, 1985).

Media and growth conditions. *H. pleuropneumoniae* forms two colony types, adherent and smooth (31, 34, 35; J. Nicolet, Ph.D. thesis, University of Bern, Bern, Switzerland, 1970). The adherent-to-smooth transition is spontaneous and apparently irreversible. Cultures frequently contain mixtures of both colony types. Thus, to study homogeneous populations, strains of *H. pleuropneumoniae* growing as an adherent-colony type were passaged in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broth containing 40 μg of NAD (Sigma Chemical Co., St. Louis, Mo.) per ml. The emergent smooth colonies were identified morphologi-

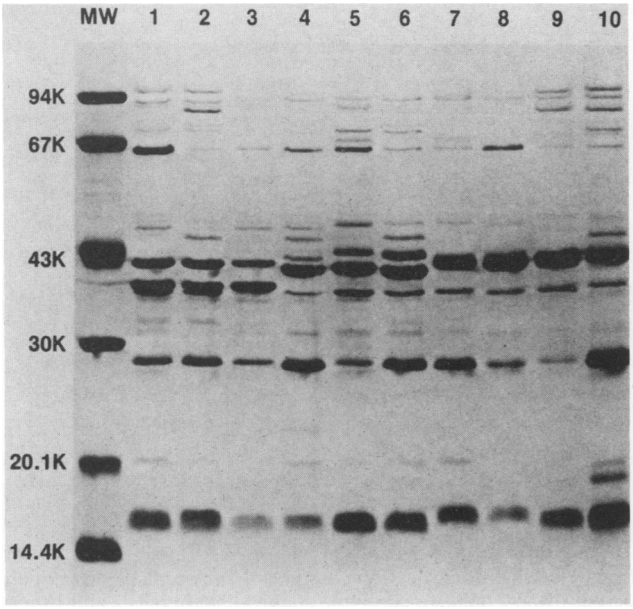

FIG. 1. SDS-PAGE profiles of Sarkosyl-insoluble OMP preparations from *H. pleuropneumoniae* reference strains of the following capsular serotypes: serotype 1, strain 4074 (lane 1); serotype 9, strains CVI 13291 and X1 (lanes 2 and 3, respectively); serotype 2, strain 1536 (lane 4); serotype 3, strain 1421 (lane 5); serotype 6, strain Femø (lane 6); serotype 4, strain M62 (lane 7); serotype 5, strain K17 (lane 8); serotype 7, strain WF83 (lane 9); serotype 8, strain 405 (lane 10). Molecular weight standards (MW) are as described in the text (94K, 94,000 molecular weight).

cally by subpassage onto a modified BHI blood agar containing 10% fresh yeast extract, 5% defibrinated horse blood, and 25 μg of NAD per ml (31). Single smooth-colony isolates were picked from each strain for subsequent evaluation.

For preparation of outer membrane-enriched fractions, smooth isolates were grown in BHI broth containing 40 μg of NAD per ml essentially as described previously (3). Cultures (5 ml) grown overnight were inoculated into 50 ml of medium in a 250-ml Erlenmeyer flask and incubated at 36°C and 200 rpm for 4 to 6 h in a shaking water bath (model G86; New Brunswick Scientific Co., Inc., Edison, N.Y.). Cells were harvested by centrifugation at 14,500 × *g* for 20 min, suspended in approximately 0.5 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4) buffer (Sigma), and stored at −70°C.

To evaluate the effect of growth medium and colony type on the resulting OMP profile, single-colony isolates of smooth and adherent-colony types were cloned from selected strains. These clones were grown in a modified PPLO broth containing 10% fresh yeast extract, 5% horse serum, 1 mg of glucose per ml, and 25 μg of NAD per ml, prepared as described previously (31) but without the agar base. This medium was designed to maintain and facilitate the handling of adherent-colony isolates. The morphology of the adherent clones grown in PPLO broth was verified at harvest by inoculation onto modified BHI blood agar.

To evaluate the effect of in vitro passage on OMP profiles, adherent and smooth clones of strain K17, the serotype 5 reference strain, were passaged 24 times in M96 medium; preparations from PPLO broth cultures were evaluated by SDS-PAGE. To evaluate the effect of in vivo passage, a pig from a respiratory-pathogen-free herd was inoculated intranasally with 2 ml of *H. pleuropneumoniae* strain 200, a serotype 5 field isolate producing adherent colonies. The culture, grown for 5 h in M96 broth, contained 7×10^8 CFU/ml. The pig was euthanized 4 days later, and the strain was reisolated from lesions of pneumonia. Preparations from PPLO broth cultures of both the original and pig-passaged isolates were evaluated by SDS-PAGE.

OMP preparation. OMP-enriched fractions were prepared essentially as described by Barenkamp et al. (3). Briefly, cells were suspended in 10 mM HEPES buffer (pH 7.4) and disrupted with a sonicator (model W225R; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) at 50% maximum output. Cells were sonicated with three to five bursts, 20 s each, with cooling in an ice bath. Whole cells and insoluble debris were removed by centrifugation at 2,500 × *g* for 20 min, and the total membrane fraction was pelleted by centrifugation at 100,000 × *g* for 60 min. The total membrane fraction was treated with 1% (wt/vol) Sarkosyl (sodium *N*-lauroyl sarcosinate; Sigma) for 30 min at room temperature to selectively solubilize the inner membrane, and the OMP-enriched fraction was pelleted by centrifugation at 100,000 × *g* for 60 min. This fraction was suspended in deionized water to a concentration of 0.5 to 2.0 mg of protein per ml and stored at −70°C. Protein concentrations were determined by the Peterson modification of the procedure of Lowry et al. (30), with bovine serum albumin (Bio-Rad Laboratories, Richmond, Calif.) as a standard.

SDS-PAGE. Discontinuous SDS-PAGE was performed with a 3.8% stacking gel and a 10% separating gel and the two-buffer system of Laemmli (13) as adapted for bacterial membranes by Ames (2). A Protean Dual 16-mm vertical slab electrophoresis cell (Bio-Rad) was used, and procedures were basically as outlined in the Hoefer catalog (Hoefer Scientific Instruments, San Francisco, Calif.). The OMP-enriched fractions were solubilized by treatment at 100°C for 5 min in sample buffer containing 2% SDS (Sigma), 5% 2-mercaptoethanol (Bio-Rad), 10% glycerol (Fisher Scientific Co., Pittsburgh, Pa.) and 0.003% bromophenol blue (Fisher) in 0.063 M Tris hydrochloride buffer (pH 6.8; Fisher). Samples were loaded at a concentration of 5 to 15 μg of protein per lane and run at a constant current of 30 mA per gel until the tracking dye was approximately 1 cm from the bottom of the gel. Bands were visualized by staining with Coomassie brilliant blue R250. Electrophoresis reagents not otherwise specified were purchased from Bio-Rad. Apparent molecular weights were calculated by comparison with standards of known molecular weight (Pharmacia Fine Chemicals, Piscataway, N.J.): phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Estimates were made from a linear regression plot of the logarithm of the molecular weight versus the electrophoretic mobility of the five lower-molecular-weight standards. For evaluation of the effect of solubilization time and temperature, samples in solubilization buffer were heated at 65 or 100°C for 30 min, in addition to the standard condition of 100°C for 5 min.

RESULTS

OMP profiles of capsular serotypes of *H. pleuropneumoniae*. Sarkosyl-insoluble, OMP-enriched fractions were prepared from smooth-colony variants of reference strains for serotypes 1 through 8 and two strains of proposed serotype 9. Three to five major bands and 10 to 20 less intensely stained bands were resolved for each strain on SDS-PAGE gels (Fig. 1). The OMP profiles were similar; however,

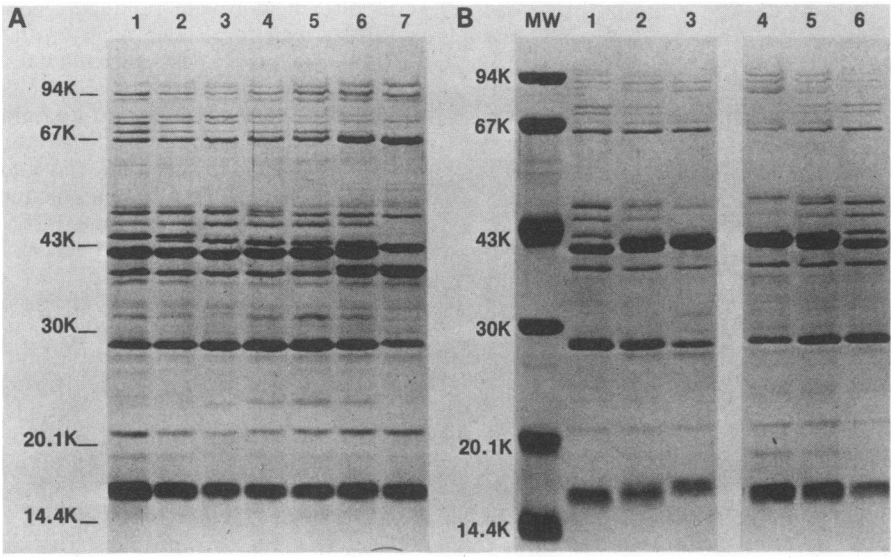

FIG. 2. Mixing experiments to confirm the nonidentity of major OMPs from reference strains of *H. pleuropneumoniae* capsular serotypes. (A) Serotype 3 (lane 1), serotypes 3 and 6 (lane 2), serotype 6 (lane 3), serotypes 6 and 2 (lane 4), serotype 2 (lane 5), serotypes 2 and 1 (lane 6), and serotype 1 (lane 7). The positions of the molecular weight standards are indicated on the left. (B) Serotype 6 (lanes 1 and 6), serotypes 6 and 5 (lane 2), serotype 5 (lane 3), serotype 7 (lane 4), and serotypes 7 and 6 (lane 5). Molecular weight standards (MW) are as described in the text.

differences were apparent in the migration of major bands in the 39,000- to 44,000-molecular-weight region and a 16,000- to 16,500-molecular-weight protein (16K to 16.5K protein).

Major 42.5K, 39K, 29K, and 16K proteins were observed in preparations from serotype 1 and serotype 9 strains (Fig. 1). A 38.5K protein was observed in preparations from serotype 1 strain 4074 and serotype 9 strain CVI 13291, but was not seen in preparations from serotype 9 strain X1. Proteins (41K, 29K, and 16K) were observed in preparations from serotype 2, 3, and 6 strains. Proteins (43.5K, 42K, and 29K) were observed in preparations from serotype 4, 5, and 7 strains. The 43.5K and 42K proteins were best resolved when less protein was applied to the gel. A 16K protein was also present in the serotype 7 preparation. In contrast, a 16.5K protein was observed in preparations from the serotype 4 and 5 strains. Serotype 8 had major 43K, 29K, and 16K proteins. A 43K doublet was apparent on some gels. Numerous less intensely stained bands (21K, 43K to 50K, and 66K to 94K) were evident in preparations from all serotypes.

The unique mobility of major OMPs was confirmed by mixing experiments to determine whether the mixture of two strains produced a composite pattern of the individual preparations. The major 41K protein from the serotype 2, 3, and 6 isolates comigrated; however, differences were apparent in the less intensely stained proteins migrating with molecular weights between 43,000 and 49,000 (Fig. 2A). The 41K band of serotype 2 could be distinguished from the 42.5K band of serotype 1, and the major 39K band of serotype 1 was distinct from the less intense 38.5K band present in the other serotypes (Fig. 2A). The mobility of the 42K band of serotypes 5 and 7 was distinguished from that of the 41K band of serotype 6 (Fig. 2B). The 43K band of serotype 8 was distinct from the 42K band of serotypes 5 and 7 (data not shown). However, the resolution was not sufficient to allow distinction of either the 42K band of serotypes 4, 5, and 7 from the 42.5K band of serotypes 1 and 9 or the 43K band of serotype 8 from the 43.5K band of serotypes 4, 5, and 7 (data not shown).

Heat-modifiable OMPs of *H. pleuropneumoniae*. The effect of solubilization time and temperature on OMP profiles was determined for all serotype reference strains and selected serotype 1, 5, and 7 field isolates. A comparison of the OMP profiles when Sarkosyl-insoluble fractions were heated in sample buffer at 65 or 100°C for 30 min in addition to 100°C for 5 min is shown in Fig. 3. The 29K band of serotypes 1, 3, 5, 7, and 9 decreased in intensity with increased time or temperature of incubation. There was a corresponding increase in staining intensity of proteins with apparent molecular weights of 42,500 (serotypes 1 and 9), 38,500 (serotype 3), and 43,500 (serotypes 5 and 7). The 29K protein from serotype 2, 4, 6, and 8 strains decreased slightly in staining intensity with increased time or temperature of incubation, but substantial quantities of the 29K proteins remained even after treatment at 100°C for 30 min. In preparations from these strains, 43.5K proteins were observed with increasing time and temperature of sample preparation. A 38.5K protein appeared in preparations from all strains after the treatment of samples at 100°C. The OMP profiles of samples heated at 100°C for 90 s, 10 min, and 15 min were essentially the same as those heated for the standard time of 5 min. It was only with treatment at 100°C for 30 min that the 29K proteins of serotypes 1, 3, 5, 7, and 9 essentially disappeared.

Seven OMP patterns were distinguished among the reference strains of the nine serotypes on the basis of differences in the mobilities of the major proteins and the heat modifiability of the 29K proteins (Table 1). The OMP profile obtained with serotype 1 and 9 strains, designated pattern 1, is characterized by major 39K and 42.5K bands. Serotype 2, 3, and 6 preparations had a major 41K band. In contrast to the 29K band of serotypes 2 and 6, the 29K band of serotype 3 was not observed after treatment at 100°C for 30 min. On this basis, serotypes 2 and 6 were assigned to pattern 2 and

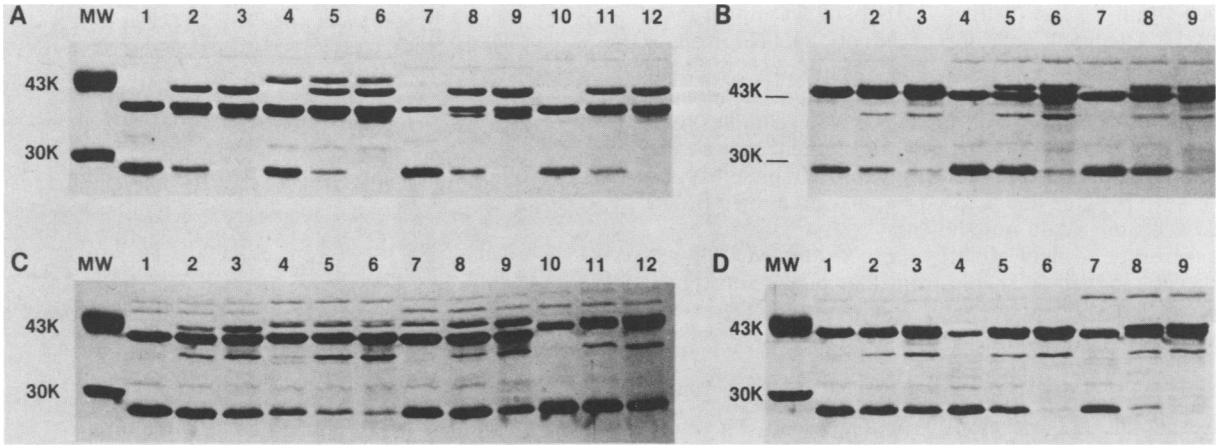

FIG. 3. Effect of solubilization time and temperature on SDS-PAGE mobility of major OMPs of reference strains of *H. pleuropneumoniae* capsular serotypes. Samples of each preparation were heated at 65°C for 30 min, 100°C for 5 min, or 100°C for 30 min and run in adjacent lanes (left to right, respectively). (A) Serotype 1 reference strain 4074 (lanes 1 through 3), serotype 1 field isolate 95 (lanes 4 through 6), serotype 9 strain CVI 13291 (lanes 7 through 9), and serotype 9 strain X1 (strains 10 through 12). (B) Serotype 5 reference strain K17 (lanes 1 through 3), serotype 5 field isolate 200 (lanes 4 through 6), and serotype 5 field isolate 119 (lanes 7 through 9). (C) Serotype 2 reference strain 1536 (lanes 1 through 3), serotype 3 reference strain 1471 (lanes 4 through 6), serotype 6 reference strain Femø (lanes 7 through 9), and serotype 8 reference strain 405 (lanes 10 through 12). (D) Serotype 4 reference strain M62 (lanes 1 through 3), serotype 7 reference strain WF83 (lanes 4 through 6), and serotype 7 field isolate 114 (lanes 7 through 9). Only the relevant portions of the gels are shown. Molecular weight standards (MW) are as described in the text.

serotype 3 was assigned to pattern 3. Serotypes 4 and 5 were distinguished from the other serotypes by a 16.5K protein. They differed from each other in the heat modifiability of the 29K band and, thus, were assigned to patterns 4 and 5, respectively. Serotype 7, with 42K and 43.5K bands, and serotype 8, with a 43K doublet were assigned patterns 6 and 7, respectively.

Effects of colony type, growth conditions, and culture passage. To assess the effect of growth medium on OMP profiles, smooth-colony variants of serotype 5 strain K17 were grown in BHI and PPLO broth, and OMP-enriched fractions were prepared. There were no differences in the OMP profiles of these preparations (Fig. 4). The effect of harvest time on the OMP profile of strain K17 was also examined. Cells were grown on BHI broth and harvested after 5, 12, or 24 h of incubation. OMP profiles of these preparations were indistinguishable (Fig. 4).

H. pleuropneumoniae forms two colony types, adherent and smooth, the latter reported to reflect partial loss of capsule (Nicolet, Ph.D thesis). However, we observed a negative image suggestive of a capsule surrounding the cells of smooth- and adherent-colony clones of 15 strains when dry India ink stains were examined by light microscopy (data not shown). The reference strains of serotypes 1 through 4, 7, and 8 and serotype 9 strain CVI 13291 were of the smooth-colony type, while the serotype 6 reference strain and serotype 9 strain X1 were adherent. The reference strain for serotype 5 formed a mixture of the two colony types. To determine whether colony type influenced OMP profiles, smooth and adherent clones of serotype 5 strain K17 were grown in PPLO medium, and Sarkosyl-insoluble fractions were prepared. No differences were observed in these preparations (Fig. 4). One serotype 1 strain, three additional serotype 5 strains, and one serotype 7 strain were examined in the same manner (data not shown). No differences in the observed OMP profiles of these strains were attributable to colony type or growth medium.

The stability of the OMP profiles during in vitro and in vivo passage was also examined. Smooth and adherent clones of strain K17 were passaged 24 times in M96 broth. No changes were observed in the OMP profiles (Fig. 4). The OMP profiles of strain 200, a serotype 5 field isolate, did not change after inoculation and reisolation from a pig (data not shown).

OMP profiles of field isolates. The profiles of smooth-colony clones from 95 *H. pleuropneumoniae* serotype 1, 5, 7, and 9 field isolates were compared with those of the reference strains for these serotypes (Fig. 5). Of 15 serotype 1 isolates examined, 7 had a major 45K band which was not evident in six extractions or repeated SDS-PAGE runs of the serotype 1 reference strain 4074. Seven isolates, four of which expressed the 45K protein, were selected for further study. Six of the seven strains were stable with respect to expression or lack of expression of this protein; however, the protein was observed only in one of three preparations from the seventh isolate. The serotype 9 field isolate examined had an OMP profile which was identical to that of strain X1 with the exception of an additional 45K band. This protein was absent in two subsequent preparations from the same strain.

The 70 serotype 5 field isolates examined had OMP profiles that were similar, but not identical, to that of

TABLE 1. OMP patterns of reference strains of *H. pleuropneumoniae* capsular serotypes

Pattern	Serotype	Major 39K to 44K OMPs	Heat-modifiability of 29K OMP[a]	Low-molecular-weight OMPs
1	1, 9	39K, 42.5K	Yes	16K
2	2, 6	41K	No	16K
3	3	41K	Yes	16K
4	4	42K, 43.5K	No	16.5K
5	5	42K, 43.5K	Yes	16.5K
6	7	42K, 43.5K	Yes	16K
7	8	43K[b]	No	16K

[a] Loss of the 29K protein after samples were treated for 30 min at 100°C.
[b] A doublet was apparent on some gels.

reference strain K17. A distinct 43.5K band was apparent in SDS-PAGE electropherograms of all of the field isolates examined. In contrast, the 42K to 43.5K doublet of strain K17 was resolved with difficulty and was most evident in gels in which less protein was applied or when samples were heated at 100°C for 30 min (Fig. 3B). Heterogeneity was also observed in the migration of the 16.5K protein of serotype 5 isolates. Two isolates had a 16K protein, and a third isolate had a 17K protein (data not shown).

Nine serotype 7 field isolates were examined. All had OMP profiles indistinguishable from that of the serotype 7 reference strain WF83.

DISCUSSION

Nicolet et al. (22) previously characterized whole-cell proteins of *H. pleuropneumoniae* by SDS-PAGE and were unable to find differences among preparations from 13 isolates representing five serotypes of the organism. Using a different gel system and OMP-enriched fractions, we were able to distinguish seven electrophoretic patterns among the nine known capsular serotypes. Designation of these patterns was based on the differences in mobility of the proteins in the molecular-weight regions of the gel between 39,000 and 44,000 and between 16,000 and 16,500 and on the heat modifiability of the 29K protein(s). Mixing experiments confirmed the distinct mobilities of the major bands used to identify the seven observed patterns.

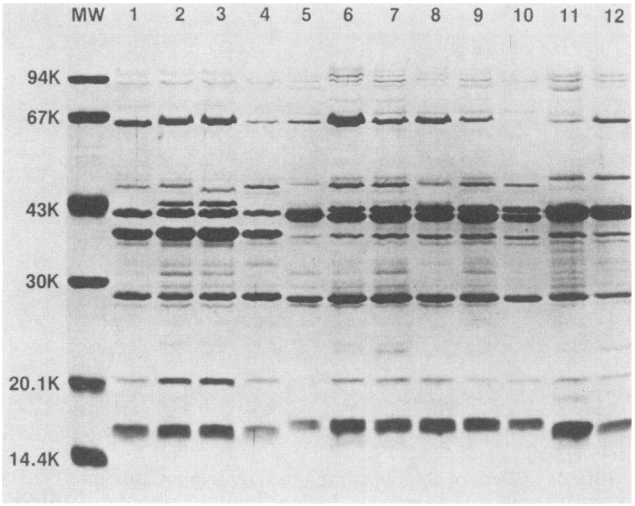

FIG. 5. SDS-PAGE profiles of OMP-enriched fractions from reference strains and field isolates of *H. pleuropneumoniae* serotypes 1, 5, and 7. Serotype 1: reference strain 4074 (lane 1) and field isolates 48, 141, and 184 (lanes 2 through 4, respectively). Serotype 5: reference strain K17 (lane 5) and field isolates (25, 49, 4, 116, and 149 (lanes 6 through 10, respectively). Serotype 7: reference strain WF83 (lane 11) and field isolate 114 (lane 12). Molecular weight standards (MW) are as described in the text.

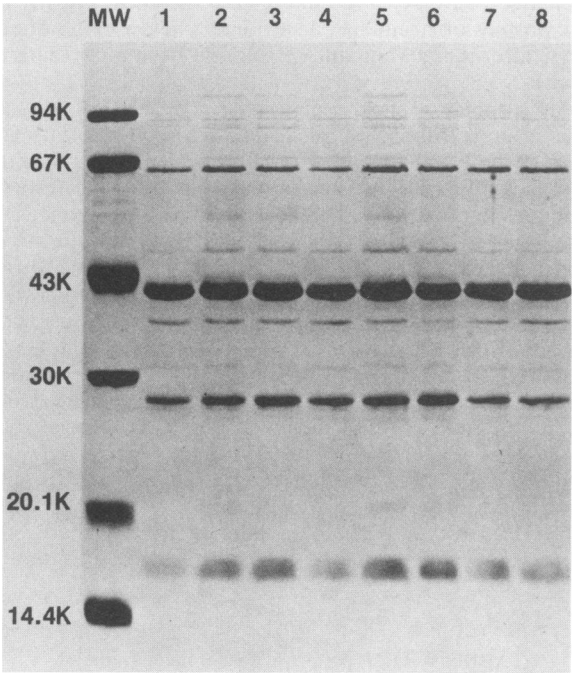

FIG. 4. Effect of in vitro passage, colony type, growth medium, and harvest time on SDS-PAGE profiles of OMP-enriched preparations from *H. pleuropneumoniae* K17, serotype 5. The following cultures were harvested after 5 h of growth: smooth-colony type, 3rd passage, grown in BHI broth (lane 1); smooth-colony type, 3rd passage, grown in PPLO broth (lane 2); adherent-colony type, 3rd passage, grown in PPLO broth (lane 3); smooth-colony type, 24th passage, grown in BHI broth (lane 4); smooth-colony type, 24th passage, grown in PPLO broth (lane 5); and adherent-colony type, 24th pasage, grown in PPLO broth (lane 6). Cultures of the smooth-colony type, 3rd passage, were grown in BHI broth and harvested after 12 h (lane 7) and after 24 h (lane 8) of growth. Molecular weight standards (MW) are as described in the text.

All of the reference strains had a 29K OMP. This band varied in intensity and, in preparations from serotypes 1, 3, 5, 7, and 9, the 29K protein was not observed after the treatment of samples at 100°C for 30 min. Concomitantly, a protein with a higher apparent molecular weight was present in samples treated at 100°C, suggesting that this protein represented the heat-modified form of the 29K protein. Both the nondenatured and heat-modified forms were present in samples treated at 100°C for 5 min; it was only after boiling for 30 min that the protein was converted completely to the heat-modified form. With most gram-negative bacteria, conversion to the heat-modified form occurs at temperatures below 100°C (4). However, the 29K protein of *H. pleuropneumoniae* resembles the heat-modifiable proteins of *Haemophilus influenzae* type b and *Pseudomonas aeruginosa*, for which extended time at 100°C is required for full conversion to the heat-modified form (6, 12, 17).

The slight decrease in the intensity of the 29K band of serotypes 2, 4, and 6 which occurred after treatment at 100°C for 30 min seemed to correspond to an increase in both the 38.5K and 43.5K bands. However, in preparations from these serotypes, the 29K band was still prominent in samples boiled for 30 min. The 29K band of these serotypes may represent a mixture of heat-modifiable and non-heat-modifiable proteins.

We have not included a comparison of the minor or less intensely stained bands in defining the SDS-PAGE patterns. Although Sarkosyl-insoluble fractions are enriched in the major OMPs (1, 5, 8), detergent treatment may result in the loss of some minor proteins (3, 5). Occasionally, variability of the minor bands of *H. pleuropneumoniae* OMP profiles was observed when different preparations of the same isolate were examined, indicating that their presence may be affected by culture or extraction procedures. The possible significance of minor proteins should not be overlooked,

however, since they could represent important epidemiologic, virulence, or antigenic markers.

Growth medium, age of culture, and in vitro passage had no effect on the OMP profile of serotype 5 strain K17. The profile of serotype 5 field isolate 200 did not change after a single pig passage. These data indicate that *H. pleuropneumoniae* OMP profiles are in general stable characteristics. Similar observations have been made with other *Haemophilus* spp. (3, 16, 19, 22, 26). Stability is important if OMPs are to be considered potentially useful epidemiologic, virulence, or immunogenic markers. *H. pleuropneumoniae* OMP patterns obtained with repeated electrophoretic runs of a preparation or with additional preparations of the same reference strain were highly reproducible.

An association of colony morphology with OMP profiles has been well documented for pathogenic *Bordetella* spp. (7, 27, 29) and *Neisseria* spp. (14, 37, 38, 40). Thus, we examined the relationship of *H. pleuropneumoniae* colony type to OMP profile. Irreversible change from adherent (or waxy) to smooth colonies may occur during in vitro passage of *H. pleuropneumoniae* (31, 34, 35; Nicolet, Ph.D. thesis). The majority of the reference strains examined in this study were already of the smooth-colony type; therefore, comparisons to each other and to field isolates could only be made for smooth-colony clones of the isolates. When grown in BHI broth, isolates of the adherent-colony type autoagglutinated and were extremely sticky, making further processing of the cells impossible. Furthermore, these cultures frequently contained spontaneous smooth variants. In modified PPLO broth, however, cultures were phenotypically smooth, yet they maintained the adherent genotype as assessed by growth on BHI agar. Our evaluation of serotype 1, 5, and 7 isolates indicated that smooth and adherent clones had identical OMP profiles when grown in PPLO broth.

Nicolet has attributed the adherent-to-smooth transition to the loss of capsular material (Nicolet, Ph.D. thesis). However, our data, based on iridescence (31) and dry India ink stains, suggest that both colony types are encapsulated. Attempts to verify the presence or absence of a capsule by transmission electron microscopic examination of antibody-stabilized, ruthenium red-stained cells have been inconclusive (V. Rapp and J. Fagerland, unpublished data). The presence or absence of pili may affect colony morphology (28, 39). However, no pili were observed by transmission electron microscopy of negative-stained *H. pleuropneumoniae* cells of either colony type (V. Rapp, unpublished data). The biochemical or morphological events responsible for the change in colony type are unknown. They are not reflected in obvious differences in either encapsulation or OMPs of the organism, but they might be related to changes in the capsule which were undetected by us or to changes in LPS. Differences in colony morphology are associated with virulence determinants of other gram-negative organisms (7, 15, 27, 28, 37), and such an association may exist for *H. pleuropneumoniae*.

OMP profiles are useful epidemiologic markers in the study of disease caused by *H. influenzae* type b and *Escherichia coli*. For the *E. coli* O:K serogroups commonly involved in extraintestinal infection (1), as well as the enteropathogenic *E. coli* strains (36), OMP profiles correlated to serotype, biotype, and other stable genetic markers of clonal relationships. Achtman et al. (1) concluded that the OMP pattern was the most suitable marker of those examined for recognizing bacterial clones. Barenkamp and coworkers examined the OMP profiles of *H. influenzae* type b and concluded that the profiles were stable properties of the strains and could be used as epidemiologic markers (3, 10). Recently, electrophoretic analysis of metabolic enzymes has confirmed the clonal nature of both the *E. coli* (25) and *H. influenzae* type b populations (20).

The similarities of the OMP profiles of reference strains of the capsular serotypes of *H. pleuropneumoniae* may reflect an evolutionary relationship. For example, serotype 6 was first identified in Denmark, where only serotype 2 had been reported for many years (Nielsen, Ph.D. thesis). The OMP profiles of these two serotype reference strains are indistinguishable. The serotype 3 strain yielded a similar profile with respect to the major 41K and 16K proteins; serologic cross-reactions between serotypes 3 and 6 have been reported (31). In contrast, although serotype 8 shares type-specific antigen(s) with both serotypes 3 and 6 (24), the serotype 8 strain has a distinct OMP profile. Serologic cross-reactions have been reported for serotypes 4 and 5 and for serotypes 4 and 7 (31). The OMP profiles of strains of these serotypes, although not identical, are similar with respect to the 42K and 43.5K proteins. The existence of serotype 9 has recently been proposed, and this serotype shares type-specific antigens with serotype 1 (Nielsen, in press; J. Nicolet, personal communication). The OMP profiles of serotype 1 and serotype 9 strains are essentially identical, again suggesting evolutionary relatedness.

Field isolates of serotype 1, a prevalent serotype in U.S. swine, are heterogeneous with respect to the 45K protein; otherwise their profiles are identical to that of reference strain 4074. The expression of the 45K protein is unstable; thus, we are unable to evaluate its significance. The serotype 1 reference strain was isolated in Argentina in the early 1960s (34), and the similarity of the more recent isolates to this strain indicates that serotype 1 strains are clonal. The single serotype 9 isolate from the United States had the pattern 1 profile indicative of serotypes 1 and 9.

The predominant *H. pleuropneumoniae* isolate from swine in the midwestern United States is serotype 5 (31). Of 70 serotype 5 isolates examined, 67 had an identical OMP profile which was similar, but not identical, to the profile of the reference strain K17. The 42K to 43.5K doublet was easily resolved in preparations from all of the field isolates but was observed only with difficulty for the reference strain K17. Strain K17 was originally isolated in the early 1960s in California, and, uniquely, it was not isolated from swine but from a lamb with lesions of arthritis (11). Three field isolates differed in the mobility of the low-molecular-mass protein. Thus, strain K17 and these three field isolates appear to be closely related, but not identical, to the predominant serotype 5 clone.

Serotype 7 was first reported in Canada and the United States in 1982 (32). Serotype 7 field isolates yielded OMP profiles indistinguishable from that of the reference strain, indicating that serotype 7 isolates represent a clonal population. Thus, the majority of haemophilus pleuropneumonia of swine in the United States is caused by three populations which appear to be clonal and which can be distinguished by both serotype and OMP profile.

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