ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 2, pp. 215-220. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 2, pp. 264-271.

Proteomic Comparative Analysis of Pathogenic Strain 232 and Avirulent Strain J of *Mycoplasma hyopneumoniae*

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Received March 7, 2008
Revision received April 2, 2008

Abstract—*Mycoplasma hyopneumoniae* is an important pathogen of pigs causing enzootic pneumonia of swine. The pathogen remains largely enigmatic as far as the host–pathogen interactions are concerned. In the present study, the protein profiles of two strains of *M. hyopneumoniae* were compared by two-dimensional gel electrophoresis and mass spectrometry. The results indicate that the major adhesin P97, the 50-kDa protein derived from P159 adhesin, and the 43-kDa cleavage product of P102 are expressed at much higher levels in the pathogenic strain 232. In contrast, the avirulent strain J switches its focus to metabolism and expresses more glyceraldehyde 3-phosphate dehydrogenase in gluconeogenesis and lactate dehydrogenase, pyruvate dehydrogenase, and phosphate acetyltransferase in the pyruvate metabolism pathway. We speculate that the avirulent strain may have developed better capabilities to cope with the rich environment during repeated inoculations. Simultaneously, the capability to infect host cells may become less important so that the adhesion-related protein genes are down-regulated.

DOI: 10.1134/S0006297909020138

Key words: *Mycoplasma hyopneumoniae*, proteomics, virulence factors

Mycoplasmas are a large group of diverse prokaryotes comprising the class *Mollicutes*. Mycoplasmas are distinguished from ordinary bacteria by their small size, lack of cell wall, and their very small AT-rich genomes [1]. Most mycoplasmas (with a few exceptions, such as *Mycoplasma penetrans*) are extracellular pathogens. The pathogen–host interactions have long been intriguing questions. Intimate contact of mycoplasmas may release toxic compounds such as peroxide and damage the vulnerable host cell membrane [2]. The adhesion of mycoplasmas to host cell surface can interfere with the functions of various membrane receptors and transporters [3]. Disruption of potassium channels of porcine tracheal ciliated cells by *Mycoplasma hyopneumoniae* leading to ciliostasis has been described [3, 4].

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia. Since *M. hyopneumoniae* does not penetrate its host's cells, it is thought that its membrane surface components and unidentified secreted agents may be critically involved in pathogenesis. The molecular mechanism of *M. hyopneumoniae* in causing porcine enzootic pneumoniae is still poorly understood. So far, only the cilium adhesin protein P97 and adhesin-like proteins have been well characterized [5, 6]. It has been observed that as the passage level is increased, *M. hyopneumoniae* strain 232 gradually showed decreased adherence with a significant decrease starting at passage 50 [4, 7]. Studies have also shown that pigs inoculated with *M. hyopneumoniae* strain J caused no lesions [8]. In an *in vitro* cell culture system for swine tracheal epithelial cells, the pathogenic *M. hyopneumoniae* was observed to bind cilia of the respiratory cells, but the non-pathogenic *M. hyopneumoniae* does not [9]. Therefore, detailed comparison of the pathogenic strain 232 with the avirulent strain J should elucidate the nature of the possible pathogenic factors. In the present study, the protein profiles of the pathogenic strain 232 and an avirulent strain J of *M. hyopneumoniae* were compared in order to identify the proteins possibly responsible for virulence.

Abbreviations: 2D-PAGE, two-dimensional gel electrophoresis in polyacrylamide gel; IEF, isoelectric focusing; MS, mass spectrometry.

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MATERIALS AND METHODS

Bacterial strains and antisera. *Mycoplasma hyopneumoniae* strain J (the type strain, ATCC 25934) was acquired from the American Type Culture Collection; *M. hyopneumoniae* strain 232 (passage 28) was a virulent strain obtained from Dr. Ross, Iowa State University [10]. The mycoplasmal strains were grown in a modified Friis broth [11] at 37°C and 80 rpm for 2-3 days and harvested at late-log phase as the indicator (phenol red) just turns to yellow. Mycoplasmas cells were harvested by centrifugation at 18,000*g* for 10 min. The cells were washed extensively (3 times) with PBS buffer (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.53) to remove the remaining medium constituents. Protein concentrations were determined with an ELISA reader (Dynatech, USA) at A_{595} using Pierce Bradford Protein Assay Kit (Pierce, USA).

Two-dimensional gel electrophoresis in polyacrylamide gel (2D-PAGE). The total mycoplasmal proteins were prepared and washed thoroughly with PBS and suspended in the lysis buffer (8 M urea, 4% CHAPS, 65 mM dithiothreitol (DTT), 5 mM tributyl phosphine, 0.5% ampholytes, and 0.002% bromophenol blue). After incubation for 15 min, the sample solution was centrifuged (12,000*g* for 30 min) and the supernatant was used for 2D-PAGE analysis. The conductivity of the sample solution was measured (approximately 120-200 μS/cm) with a CDM210 conductivity meter (MetaLab, USA).

The isoelectric focusing (IEF) was performed in a IPGphore apparatus (Pharmacia, USA) and SDS-PAGE in a SE600 Ruby gel apparatus (Hoefer, USA). In brief, the protein samples of 350 μl (containing 170-600 μg protein) were loaded onto the strip (17 cm, p*I* 3-10; Bio-Rad, USA), covered with 1 ml cover oil, and subjected to IEF at 20°C (50 V, 16 h for active rehydration; 250 V, 40 min; 10,000 V, 8 h until 50,000 V-h). After the focusing step, the strips were treated with 3 ml SDS equilibration buffer (6 M urea, 30% (v/v) glycerol, 2% SDS (w/v), 0.2% DTT, and 50 mM Tris-HCl, pH 8.8) for 15 min before SDS-PAGE analysis. The IPG strips were run vertically onto the 12% SDS-PAGE gels. After electrophoresis, the gels were silver stained or Coomassie blue stained. In the silver staining process using the Silver Stain Plus Staining Kit (Bio-Rad), the gels were incubated for 30 min first with the fixing solution (40% ethanol, 1.7 M acetic acid) and then for 30 min in the sensitizing solution (30% ethanol, 0.125% glutaraldehyde, 0.2% sodium thiosulfate, 0.83 M sodium acetate). After washing three times with distilled H_2O, the gels were silver stained and developed [12]. To get better resolution in MS (mass spectrometry) analysis, only the Coomassie blue stained gels using the Bio-Rad Coomassie Brilliant Blue R-250 Staining Solutions Kit were used in protein spot identification. The gel maps were analyzed with the ImageMaster program (2D Platinum Software version 5.0; Amersham, USA).

In-gel digestion. Protein spots were excised from the Coomassie blue stained gels and in-gel digested with trypsin. The gel pieces were dried by dehydration in acetonitrile and incubated in 25 mM ammonium bicarbonate containing 10 mM DTT for 30 min at 56°C. After cooling to room temperature, the DTT solution was replaced with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 30 min at room temperature in the dark. The gel pieces were washed two times with 50 mM ammonium bicarbonate and acetonitrile (1 : 1), dehydrated by addition of acetonitrile, swollen in 100 mM ammonium bicarbonate, dehydrated in acetonitrile, and vacuum dried. The gel pieces were swollen in digestion buffer containing 25 mM ammonium bicarbonate and 20 ng/μl of porcine trypsin (Sigma, USA) at 37°C. After 30 min, the supernatant was removed and replaced with the same buffer (but without trypsin) and incubated at 37°C overnight. Peptides were extracted and concentrated two times with acetonitrile and 0.1% TFA (trifluoroacetic acid) (1 : 1) in order to get better MALDI spectra.

MALDI-TOF-mass spectrometry and database search. MALDI spectra were acquired using an autoflex time-of-flight mass spectrometer (Bruker Daltonics, Germany) equipped with a 337-nm nitrogen laser. Spectral data were obtained in the reflectron mode with an acceleration voltage of 19 kV. Each mass spectrum was derived from 50 summed scans. α-Cyano-4-hydroxy-cinnamic acid solution (50 mM) in a solvent mixture of acetonitrile and 0.1% TFA (1 : 1) was used as a MALDI matrix. The MS analysis of each protein digest was conducted immediately after the in-gel digestion. An aliquot (0.5 μl) of the sample solution was mixed with an equal volume of the matrix solution and then applied on the target plate and dried before MALDI-MS analysis. The spectra were calibrated externally using a peptide mixture that contained angiotensin I and II, substance P, fragments of adrenocorticotropic hormone (1-17) and (18-39), and bombesin.

The peptide ion mass spectra were searched against the NCBInr (http://www.ncbi.nlm.nih.gov) database using the Mascot (http://www.matrixscience.com) algorithm. Peptide masses obtained from the digestion of keratin and trypsin were excluded from the MS data before the analysis. The database searching was done by restricting the taxonomic category to bacteria (Eubacteria). The maximum number of missed cleavages was set at one. Mass accuracy was set at 150 ppm. Other parameters included enzyme (trypsin), peptide charge (1+), and variable modifications (carbamidomethyl-Cys, oxidized Met). The top matched protein having a MOWSE score above the threshold value with $p < 0.05$ was considered as positive identification.

RESULTS

The goal of the present study was to identify the proteins that differed most between the two mycoplasmal

strains, so that we might be able to gain some insights about the nature of the virulence factors. The total soluble proteins of *M. hyopneumoniae* strain J and strain 232 were separated by 2D-PAGE followed by MS analysis. The 2D-PAGE experiments and MS analysis were repeated more than three times to achieve high degree of reproducibility. The silver-stained gels usually resolved more than 300 protein spots, while the Coomassie blue stained gels resolved a few less in both of the mycoplasmal strains (data not shown). The number of the predicted coding sequences for strain 232 and strain J are 691 and 679, respectively [13, 14]. Since it is usually easier to obtain more reliable MS spectra from the Coomassie blue stained proteins, we used Coomassie blue to stain the gels until the most prominent protein spots can be clearly visualized. As shown in Fig. 1, the 2D protein profiles indicate that the major differences between strain 232 and strain J reside in four regions A-D. Strain 232 was found to express more proteins of high molecular weight (75-150 kDa) near p*I* 9-10 (region C), and proteins of approximately 50 kDa spanning from p*I* 7 through p*I* 10 (region D). On the other hand, strain J expressed more proteins of approximately 100 kDa in the vicinity of p*I* 5-6 (region A) and several proteins of 35-40 kDa near the region of p*I* 7.0-7.5 (region B). These prominent proteins were chosen as the focus of the study. The protein spots were excised for in-gel digestion experiments and subsequent MALDI-TOF MS analysis. Protein spots with Mowse score >100 (according to the probability based Mowse score, protein scores greater than 76 were considered significant, with $p < 0.05$) are included in table. As shown in Fig. 2 (c and d) and table section 1, the proteins expressed at relatively higher levels in strain 232 include: spots 232-C-1 to 232-C-4 (all were identified as the cilium adhesion-derived proteins) with apparent molecular weights of 130, 100, 100, and 68 kDa, respectively [6, 15-18]. Spots 232-D-5 and 232-D-6 with apparent molecular weight of 50 kDa and different p*I* were identified as the cleaved products of P159 protein, which was derived from mhp494 [5]. Spot 232-D-7 with apparent molecular weight of 43 kDa was identified as a protein derived from hypothetical protein P102 [17]. The experimentally determined molecular weights and p*I* of spots 232-C-1 to 232-C-4 and 232-D-5 to 232-D-7 are different from the theoretical predictions, representing the possible extensive and variable post-translational modifications among these proteins.

Different proteins were more abundant in the non-pathogenic strain J. As shown in Fig. 2 (a and b) and table section 2, spot J-A-1 with score 66 failed to have a clear identification. Spots J-A-2a to J-A-2c with fairly high

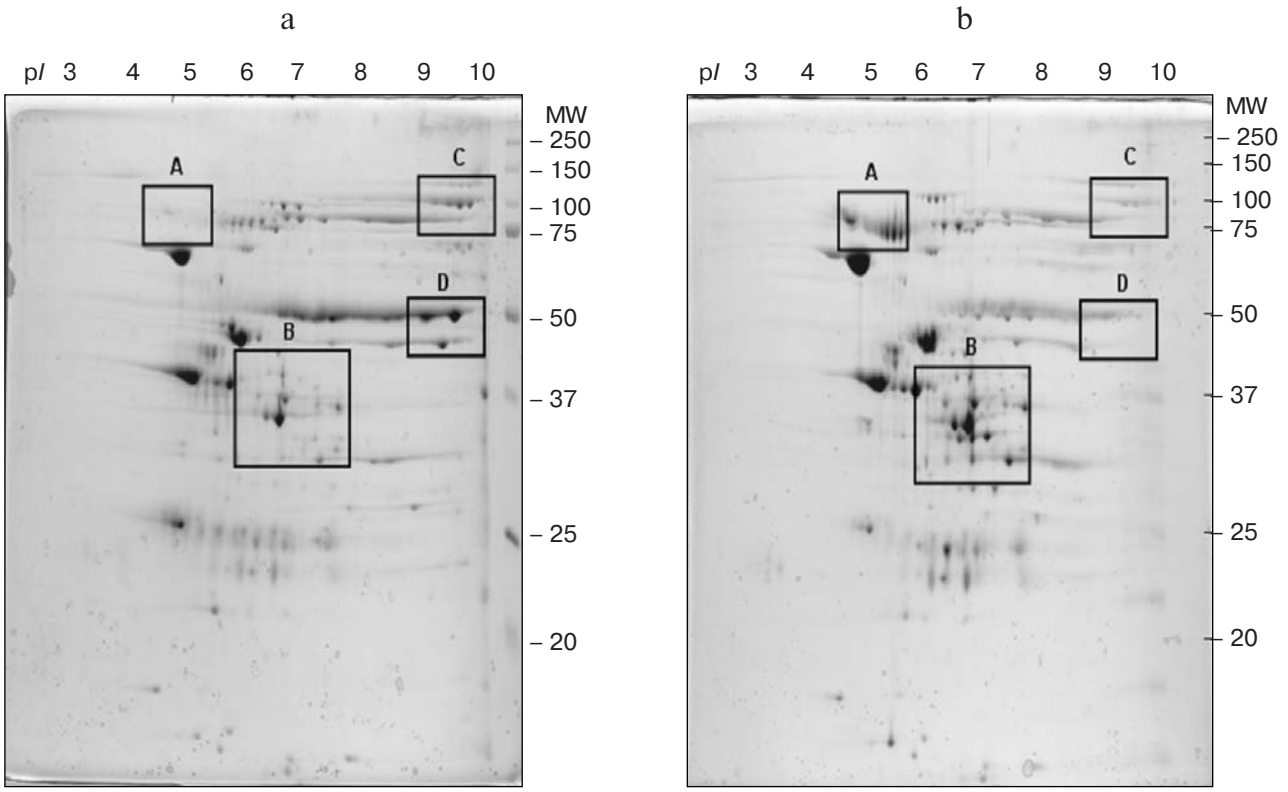

Fig. 1. Comparison of protein profiles of (a) *M. hyopneumoniae* strain 232 and (b) *M. hyopneumoniae* strain J. The 2D gels were separated by IEF using 17-cm IPG strips (p*I* 3-10), followed by SDS-PAGE on 12% gels and stained with Coomassie Brilliant Blue R250. The protein molecular weight markers (kDa) are shown on the right of the gels.

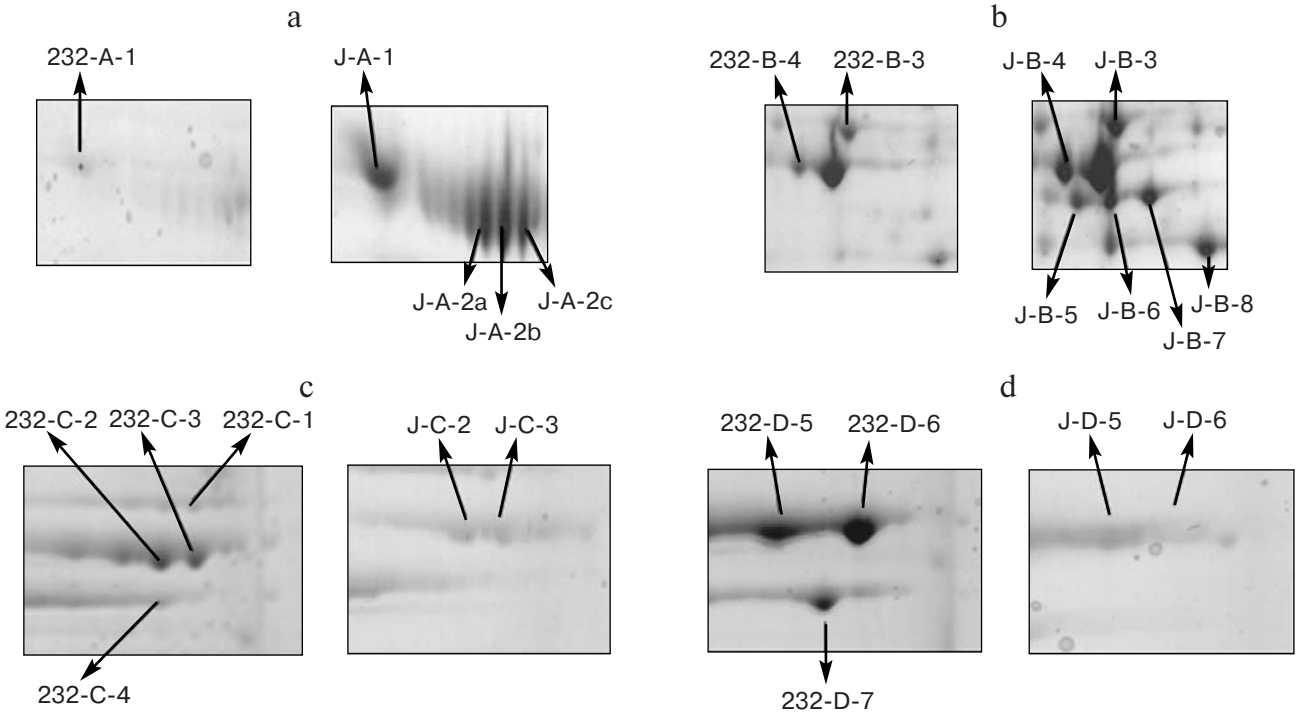

Fig. 2. Major different regions A-D (panels (a)-(d), respectively) between *M. hyopneumoniae* strain 232 (left) and strain J (right). The prominent protein spots were excised for further in-gel digestion and MS studies.

Mowse scores were tentatively identified as ch4 and secreted swine IgM, since the search for J-A-2 in the Eubacteria taxonomic database failed and the experimental molecular weight values also differed from the theoretical values. Spot J-B-3 was identified as phosphate acetyltransferase (EC 2.3.1.8) and spot J-B-4 as pyruvate dehydrogenase (EC 1.2.4.1). Spots J-B-5 to J-B-7 were identified as glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). Spot J-B-8 was identified as L-lactate dehydrogenase (EC 1.1.1.27). The Mowse scores, sequence coverage, and peptide matching are reasonably high for protein spots J-B-3 to J-B-8. The experimentally determined molecular weight values and p*I* of spots J-B-3 to J-B-8 are also in good agreement with the theoretical predictions.

DISCUSSION

Proteomic comparison of the pathogenic strain 232 with the avirulent strain J of *M. hyopneumoniae* should reveal the nature of some virulence factors. The focus of the present study is on the prominent proteins, which are expressed very differently between the pathogenic strain 232 and the avirulent strain J. The protein profiles (as shown in Fig. 1 and table) indicate that the nonpathogenic strain J produced higher amounts of metabolism-related enzymes including glyceraldehyde 3-phosphate dehydrogenase converting glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate, lactate dehydrogenase converting lactate to pyruvate, pyruvate dehydrogenase converting pyruvate to S-acetyl dihydrolipoamide and then to acetyl-CoA, and phosphate acetyltransferase converting acetyl-P to acetyl-CoA. These proteins are the key enzymes along the pyruvate metabolism pathway leading eventually to the citric acid cycle to release the energy from the breakdown of glucose molecules.

We speculate that the avirulent strain may have developed better capabilities to cope with the rich environment during the period of repeated inoculations. Simultaneously, the capability to infect host cells may become less important, so the expression of adhesion-related protein genes may be down-regulated, as those genes were not deleted in strain J [14, 15].

The apparent p*I* values of J-A-2 spots are similar to the theoretical value 5.74, but the apparent molecular weight (76 kDa) is much higher than the theoretical value (49 kDa), making the identification rather difficult. Though the contamination of mycoplasmal preparations by growth medium constituents has been described [19], we have observed that J-A-2 spots only appear repeatedly in the protein profile of strain J. In addition, the protein samples of both strains were washed more than three times with PBS to remove any contaminants from the growth medium.

The adhesion-related proteins associated with the pathogenesis process of *M. hyopneumoniae* were expressed

Comparison of the protein profiles of *M. hyopneumoniae* strain 232 and strain J. The Mr/p*I*, sequence coverage, and peptide matched for each chosen protein were estimated

Spot number	Matching protein name and accession number	Mowse score	Theoretical and apparent Mr/p*I* values	Sequence coverage, %	Peptide matched
		1. Identification of the proteins expressed more in strain 232			
232-C-1	cilium adhesin [*M. hyopneumoniae* 232] GI:54020389	132	124827/8.92 130000/9.4	28	25/40
232-C-2 232-C-3	cilium adhesin [*M. hyopneumoniae* 232] GI:54020389	105	124827/8.92 100000/9.2; 9.4	22	14/22
232-C-4	cilium adhesin [*M. hyopneumoniae* 232] GI:54020389	132	124827/8.92 68000/9.2	28	25/40
232-D-5 232-D-6	P159 derived from mhp494 [*M. hyopneumoniae* 232] GI:54020544	180	158581/8.50 50000/8.7; 9.0	19	30/41
232-D-7	conserved hypothetical protein, p102 paralog [*M. hyopneumoniae* 232] GI:53987827	147	134552/7.23 43000/8.9	13	24/31
		2. Identification of the proteins expressed more in strain J			
J-A-1	unknown	<75	– 88000/6.9	–	–
J-A-2	ch4 and secreted domain of swine IgM GI:1236646	120	49268/5.74 76000/5.7; 5.8; 5.9	53	13/38
J-B-3	phosphate acetyltransferase [*M. hyopneumoniae* 232] GI:53987727	185	35096/6.25 37000/6.9	45	18/27
J-B-4	pyruvate dehydrogenase (lipoamide) e1-β chain [*M. hyopneumoniae* 232] GI:54020422	249	36711/6.21 34000/6.7	45	26/34
from J-B-5 to J-B-7	glyceraldehyde 3-phosphate dehydrogenase [*M. hyopneumoniae* J] GI:71851517	191	36867/6.40 33000/6.8; 6.9; 7.1	49	18/30
J-B-8	L-lactate dehydrogenase [*M. hyopneumoniae* J] GI:71851616	145	34217/7.63 32000/7.3	29	15/25

at higher levels in the pathogenic strain 232. The cilium adhesin gene contains six paralogs in the genome, but only the surface protein P97 enables *M. hyopneumoniae* to attach to the cilia of the respiratory epithelium [15, 18]. Dwelling in the same operon with *P97*, *P102* gene also has six paralogs. Previous studies indicated that P102 is expressed during the disease, distributed in the extracellular matrix not cell-associated. Anti-P102 antibodies were reported to recognize P102 and its cleavage products of 72 and 42 kDa. However, the functions of P102 and its derived proteins were unknown [7, 13, 18]. Now the spot 232-D-7 with apparent molecular weight of 43 kDa was identified as a P102-derived protein. The more pronounced expression of the 43-kDa protein in the pathogenic strain 232 reveals its potential roles in bacterial virulence. For the cilium adhesin P97, it undergoes a very complex process occurring at different frequencies within multiple sites and is strain-specific [17]. Genomic comparisons reveal that P97 of *M. hyopneumoniae* 7448, 232, and J strains has 10, 15, and 9 copies of RR1 tandem repeats, respectively [14]. This variation was suggested to be one of the key determinants in different pathogenic properties of the *M. hyopneumoniae* strains [14]. In the present study, spots 232-C-1 to 232-C-4 with different molecular weights and p*I* values were all identified as cilium adhesion proteins. Their apparent molecular weight values of 130, 100, 100, and 68 kDa are very close to those of the predicted cleaved products of 124, 97, and 70 kDa of adhesin [17, 20]. Protein 232-C-4 also correlates well with the predicted P97 (observed molecular weight 65.4 kDa and p*I* 8.8) of *M. hyopneumoniae* strain 7448, but with a much lower Mowse score [20]. How the cleavage processes are regulated and whether these cleavage products can alter the bacterial surface architecture and exert their functional roles still awaits further studies.

Another set of proteins expressed at higher levels in the pathogenic strain 232 are spot 232-D-5 and 232-D-6 (molecular weight ~50 kDa). They were identified as one of the cleavage products of the P159 membrane protein, P52, with varied p*I* values. P159 is a proteolytically processed surface adhesin of *M. hyopneumoniae* consisting of P110 (110 kDa), P52 (52 kDa), and P27 (27 kDa) subunits [5]. The corresponding P76 protein (observed molecular weight 58.1 kDa and p*I* 9.1) of *M. hyopneumoniae* strain 7448 has also been reported recently [20].

In conclusion, the comparative analysis of the protein profiles of the two strains of *M. hyopneumoniae* has shown that strain J produces relatively higher amounts of metabolism-related proteins, while the P97 adhesin, the cleavage product P50 of P159 adhesin, and the P102 associated protein of previously unknown function were expressed at much higher levels in the pathogenic strain 232. The results imply that differential gene expression may explain partially the difference in pathogenicity between the two strains, since both of the strains carry the same set of adhesin-related genes [15]. However, other than the variation of the tandem repeats RR1 and RR2 of P97 adhesin in different strains [14], we are short of knowledge about how the pathogenesis-related genes are regulated and cause the differential expression patterns. In addition to the prominent proteins that we have elucidated in the present study, identification and functional studies of some less abundant proteins, low molecular weight proteins, and even the cleaved peptides should be the focus of future investigations.

This work was supported in part by grants from the National Science Council ROC (NSC93-2311-B259-003 and NSC94-2311-B259-005).

REFERENCES

1. Razin, S., Yogev, D., and Nato, Y. (1998) *Mol. Biol. Rev.*, **62**, 1094-1156.
2. Almagor, M., Kahane, I., Gilon, C., and Yatziv, S. (1996) *Infect. Immun.*, **52**, 240-244.
3. Izutsu, K. T., Fatherazi, S., Belton, C. M., Oda, D., Cartwright, F. D., and Kenny, G. E. (1996) *In vitro Cell Dev. Biol. Anim.*, **32**, 361-365.
4. Debey, M. C., and Ross, R. F. (1994) *Infect. Immun.*, **62**, 5312-5318.
5. Burnett, T. A., Dinkla, K., Rohde, M., Chhatwal, G. S., Uphoff, C., Srivastava, M., Cordwell, S. J., Geary, S., Liao, X., Minion, F. C., Walker, M. J., and Djordjevic, S. P. (2006) *Mol. Microbiol.*, **60**, 669-686.
6. Zhang, Q., Young, T. F., and Ross, R. F. (1995) *Infect. Immun.*, **63**, 1013-1019.
7. Zhang, Q., Young, T. F., and Ross, R. F. (1994) *Infect. Immun.*, **62**, 1616-1622.
8. Zielinski, G. C., and Ross, R. F. (1990) *Am. J. Vet. Res.*, **51**, 344-348.
9. Young, T. F., Thacker, E. T., Erickson, E. Z., and Ross, R. F. (1999) *Vet. Microbiol.*, **71**, 269-279.
10. Bereiter, M., Young, T. F., Joo, H. S., and Ross, R. F. (1990) *Vet. Microbiol.*, **25**, 177-192.
11. Muhlradt, P. F., Kiess, M., Meyer, H., Sussmuth, R., and Jung, G. (1998) *Infect. Immun.*, **66**, 4804-4810.
12. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.*, **68**, 850-858.
13. Minion, F. C., Lefkowitz, E. J., Madsen, M. L., Cleary, B. J., Swartzell, S. M., and Mahairas, G. G. (2004) *J. Bacteriol.*, **186**, 7123-7133.
14. Vasconcelos, A. T., Ferreira, H. B., et al. (2005) *J. Bacteriol.*, **187**, 5568-5577.
15. Hsu, T., Artiushin, S., and Minion, F. C. (1997) *J. Bacteriol.*, **179**, 1317-1323.
16. Wilton, J. L., Scarman, A. L., Walker, M. J., and Djordjevic, S. P. (1998) *Microbiology*, **144**, 1931-1943.
17. Djordjevic, S. P., Cordwell, S. J., Djordjevic, M., Wilton, A. J., and Minion, F. C. (2004) *Infect. Immun.*, **72**, 2791-2802.
18. Adams, C., Pitzer, J., and Minion, F. C. (2005) *Infect. Immun.*, **73**, 7784-7787.
19. Nicolet, J., Paroz, P., and Kristensen, B. (1980) *J. Gen. Microbiol.*, **119**, 17-26.
20. Pinto, P. M., Chemale, G., de Castro, L. A., Costa, A. P., Kich, J. D., Vainstein, M. H., Zaha, A., and Ferreira, H. B. (2007) *Vet. Microbiol.*, **121**, 83-93.