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Cleavage of Influenza A Virus H1 Hemagglutinin by Swine Respiratory Bacterial Proteases

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Cleavage of influenza A virus hemagglutinin (HA) is required for expression of fusion activity and virus entry into cells. Extracellular proteases are responsible for the proteolytic cleavage activation of avirulent avian and mammalian influenza viruses and contribute to pathogenicity and tissue tropism. The relative contributions of host and microbial proteases to cleavage activation in natural infection remain to be established. We examined 23 respiratory bacterial pathogens and 150 aerobic bacterial isolates cultured from the nasal cavities of pigs for proteolytic activity. No evidence of secreted proteases was found for the bacterial pathogens, including *Haemophilus parasuis*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, and *Streptococcus suis*. Proteolytic bacteria were isolated from 7 of 11 swine nasal samples and included *Staphylococcus chromogenes*, *Staphylococcus hyicus*, *Aeromonas caviae*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Enterococcus* sp. Only *P. aeruginosa* secreted a protease, elastase, that cleaved influenza virus HA. However, compared to trypsin, the site of cleavage by elastase was shifted one amino acid in the carboxy-terminal direction and resulted in inactivation of the virus. Under the conditions of this study, we identified several bacterial isolates from the respiratory tracts of pigs that secrete proteases in vitro. However, none of these proteolytic isolates demonstrated direct cleavage activation of influenza virus HA.

Cleavage of the influenza A virus hemagglutinin (HA) is required for cell entry by receptor-mediated endocytosis (19, 20, 22, 27). During viral entry, the HA undergoes a conformational change within the acidic environment of the endosome (4, 6, 32, 41). Cleavage of the intact HA0 into HA1 and HA2 allows exposure of the amino-terminal fusion peptide of the HA2 within the endosome, resulting in fusion of the viral envelope with the endosomal membrane. The importance of HA cleavage in viral pathogenicity and tissue tropism has recently been reviewed (25, 27).

Susceptibility of HA to cleavage by host intracellular proteases is the major factor distinguishing highly virulent avian influenza viruses from avirulent avian and mammalian viruses (3, 12, 18, 26). The HAs of virulent avian viruses contain the multiple basic amino acid sequence R-X-K/R-R, making them susceptible to host intracellular proteases, such as furin and PC6 (13, 14, 37, 39, 40). The presence of these proteases in virtually all tissues contributes to the systemic spread and high virulence of these viruses in birds. In contrast, the HAs of avirulent avian and mammalian viruses are not susceptible to cleavage by these intracellular proteases and are predominantly cleaved by extracellular serine proteases. Early studies clearly demonstrated the requirement for trypsin in cleavage activation of influenza A viruses in vitro (19, 20, 22). Subsequently, a protease in the allantoic fluid, similar to the mammalian clotting factor Xa, was shown to activate influenza virus in embryonated chicken eggs (9). This protease enables replication in allantoic membranes and confers specific tissue tropism in eggs (9, 28).

In contrast, the source of specific proteases that confer cleavage activation during natural infection of mammalian hosts has not been fully determined. Respiratory tract proteases capable of cleavage activation of influenza virus have been recovered from nasal wash specimens of children with upper respiratory disease (2). Several endogenous host inflammatory and hemostasis proteases, such as kallikrein, urokinase, thrombin, and plasmin, cleave the HAs of some, but not all, influenza viruses (30). Other host proteases, such as granulocyte proteases and cathepsin D, cleave the HA but do not confer infectivity to the virus, suggesting that cleavage occurs at an inappropriate site (2). Tryptase Clara, a trypsin-like protease produced by rat bronchiolar Clara cells, is secreted into the rat respiratory tract and is capable of cleavage activation of influenza viruses (17). Similar proteases have not been demonstrated in other mammalian species. Thus, the specific role that these or other host proteases play in influenza virus cleavage activation within the respiratory tract has not been conclusively determined.

Bacteria present in the respiratory tract are potential sources of proteases that could contribute to cleavage activation of influenza virus in vivo (1, 30, 34, 35). Proteases from some strains of *Streptomyces griseus*, *Staphylococcus aureus*, and *Aerococcus viridans* secrete proteases that directly cleave the HAs of several, but not all, influenza A viruses (19, 30, 34, 35). HA cleavage by *S. aureus* and *A. viridans* proteases confers virus infectivity and replication in vitro and augments virus replication and pathogenesis in mice (30, 34, 35). Alternatively, staphylokinase, streptokinase, and a protease from *Serratia marcescens* can facilitate cleavage activation by generation of plasmin from plasminogen (1, 30).

The contributions of endogenous and microbial proteases in natural influenza virus infection remain to be established. Influenza remains a particularly important respiratory disease in humans, as well as pigs. Clinically, the diseases are remarkably similar in both species and are typically restricted to upper respiratory tract infections. Thus, pigs provide an excellent model with which to study influenza. In this project, clinical isolates of pathogenic respiratory bacteria from pigs with re-

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spiratory disease (respiratory bacterial pathogens) and commensal nasal bacteria (nasal flora) from pigs were examined for their ability to secrete proteases that cleave the HA of a swine influenza virus. While none of the pathogens secreted proteases, we identified several proteolytic isolates from the nasal cavity. Of these, only *Pseudomonas aeruginosa* produced a protease that cleaves the HA. The *Pseudomonas* protease that cleaves the HA was determined to be elastase (pseudolysin [EC 3.4.24.26]). However, *Pseudomonas* elastase cleaved the HA at a site resulting in decreased, rather than increased, virus infectivity.

MATERIALS AND METHODS

Virus. A laboratory-derived high-growth reassortant virus created by combination between A/Swine/WI/1915/88 (H1N1) and A/PR8/34 (H1N1) was used for the HA cleavage and the influenza virus activation experiments (SW/WI HG). This virus contains the HA and neuraminidase (NA) of A/Swine/WI/1915/88 and was used for these experiments because it grows to high titers in both eggs and tissue culture.

Preparation of uncleaved SW/WI HG virus stock. MDCK cells were infected with egg-grown SW/WI HG at a multiplicity of infection of 1 to 5 in minimal essential medium (MEM). After an initial incubation of 1 h, the medium was replaced with fresh MEM, and incubation continued for an additional hour. Cells were then washed with 0.9% NaCl at pH 3 to neutralize the remaining extracellular input virus, followed by two washes with Dulbecco's phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ [pH 7]). Fresh MEM was exchanged two additional times at hourly intervals, and the infected cells were incubated overnight in MEM. Cell supernatants containing virus with uncleaved HA were harvested and stored at −70°C.

***P. aeruginosa* proteases and protease-specific antisera.** Purified *P. aeruginosa* elastase (pseudolysin [EC 3.4.24.26]) and alkaline protease (serralysin [EC 3.4.24.40]) were obtained from Nagase & Co., Ltd, Tokyo, Japan. Protease-specific rabbit antisera were prepared by the Polyclonal Antibody Service, Animal Care Unit of the University of Wisconsin Medical School. *Pseudomonas* elastase and alkaline protease were inactivated according to the method of Homma et al. (11) prior to injection into rabbits for antibody production.

Bacterial isolates. Swine respiratory bacterial pathogens were obtained from the Iowa State University Veterinary Diagnostic Laboratory and the University of Wisconsin Veterinary Diagnostic Laboratory. All respiratory bacterial pathogens were isolated from postmortem respiratory tract cultures from pigs with respiratory disease. Isolates were stored at −70°C. The pathogenic bacteria included eight *Haemophilus parasuis*, five *Pasteurella multocida*, four *Actinobacillus pleuropneumoniae*, three *Bordetella bronchiseptica*, and three *Streptococcus suis* isolates. One *P. multocida* isolate and five *H. parasuis* isolates were obtained from pigs that died of combined influenza virus and bacterial pneumonia at the University of Wisconsin Arlington Animal Research Facility.

Bacteria present in the nasal cavities of 3- to 10-week-old healthy pigs were cultured aerobically from nasal swab or nasal wash specimens. The pigs were obtained from commercial production facilities and were housed indoors on concrete for at least 3 days prior to sample collection. Nasal swabs were obtained by insertion of a Dacron swab approximately 4 to 6 cm into the nasal cavities of awake or anesthetized pigs ($n = 8$). Nasal washes were obtained by lavage of the nasal cavities of anesthetized pigs ($n = 3$) with 10 ml of sterile PBS. Samples were transported promptly to the laboratory and inoculated onto a tryptic soy agar plate supplemented with 5% sheep blood (Remel, Lenexa, Kans.) and a chocolate agar plate. Both plates were streaked for isolation and incubated in 5% CO_2 at 37°C. One or more isolates were selected from each distinct colony morphology type observed. Five to 25 isolates were obtained from each sample and stored in tryptic soy broth (TSB) with 10% glycerol at −70°C. No isolate was passaged more than three times prior to storage. A total of 150 bacterial isolates were screened for proteolytic activity. All isolates demonstrating protease activity by casein agar assay were further identified to the genus and species level at the University of Wisconsin Veterinary Diagnostic Laboratory.

Two additional laboratory isolates were used as reference controls in the study: *P. aeruginosa* PAO1 and a *Bacillus* sp. strain (not *B. subtilis* or *B. cereus*) isolated from Sigma technical-grade casein. *P. aeruginosa* PAO1 is a wound isolate that has been well characterized and is considered to be the prototypical strain (10). It produces elastase and alkaline protease in addition to several other secreted virulence factors. Preliminary tests of the *Bacillus* sp. isolate showed it produces a protease that cleaves the HA and activates influenza virus in vitro. These organisms were used for comparison throughout the experiments. All bacterial isolates are available upon request from the corresponding author (R.J.C.).

Preparation of bacterial supernatants. Stored bacterial isolates were cultured on blood or chocolate agar plates overnight. All pathogenic bacteria and the first 50 nasal cavity isolates were transferred to 10 ml of three different broth media: brain heart infusion broth (BHIB; Difco Laboratories, Detroit, Mich.), dialyzed BHIB, and tryptic soy broth (Sigma). The dialyzed BHIB was prepared by dialyzing 2× concentrated BHIB through dialysis tubing with a molecular weight cutoff of 6,000 to 8,000 (Spectra/Por 1; Fisher Scientific, Itasca, Ill.) against H_2O. After dialysis, electrolytes and H_2O were added to the concentrations reported by the manufacturer; this was done to remove soluble amino acids and carbohydrate that might inhibit protease production for some organisms. Some of the pathogenic isolates required the addition of IsoVitaleX enrichment (Becton Dickinson Microbiology Systems, Cockeysville, Md.) for growth. The broth cultures were incubated for 48 to 60 h at 37°C on a rotator wheel. The cultures were clarified by centrifugation (12,000 × *g* for 10 min at 4°C) and filtered through a μStar 0.22-μm-pore-diameter syringe filter (Costar; Corning, Boston, Mass.). The samples were then concentrated to approximately 1 ml by ultrafiltration with a Centriprep-10 concentrator (Amicon, Danvers, Mass.).

The remaining 100 normal flora isolates were first screened by streaking on caseinate agar plates consisting of 3 g of Hammerstein casein (ICN Biochemicals, Inc., Aurora, Ohio) per liter, 7 g of peptonized milk (Unipath Ltd, Basingstoke, Hampshire, United Kingdom) per liter, and 12 g of special agar (Unipath Ltd) per liter. Cultures were examined at 24 and 48 h for proteolytic activity, observed as a zone of clearing around individual colonies. Positive isolates were cultured in TSB, and culture supernatant concentrates were prepared as described above.

Detection of proteolytic activity in bacterial supernatants. The concentrated culture supernatants were tested for proteolytic activity on casein agar plates. The casein agar consisted of 25 mM Tris (pH 7.2), 150 mM NaCl, 0.6% casein (Sigma technical grade), and 1% Bacto agar (Difco). Aliquots (10 μl) of concentrated culture supernatants were placed in 3-mm-diameter wells cut in the casein agar and incubated at 37°C for 18 h. The plates were overlaid with 3% acetic acid, and proteolytic activity was noted as a zone of clearing around the sample well. Tolylsulfonyl phenylalanyl chloromethyl ketone trypsin (Worthington Biochemical Corp., Freehold, N.J.) was used as a positive control standard at a concentration of 10 μg/ml. Caseinolytic activity, relative to trypsin, was determined by measuring the diameter of the proteolytic zones around each well. Samples with caseinolytic activity were also tested with the PepTag protease detection assay (Promega, Madison, Wis.) according to the manufacturer's instructions.

Assay of HA cleavage activity. Bacterial supernatants with proteolytic activity were tested for their ability to cleave influenza virus HA by digestion of radioimmunoprecipitated HA. Radioimmunoprecipitation was performed according to the method of Kida et al. (16). MDCK cells were infected with SW/WI HG at a multiplicity of infection of 1. After 1 h, inoculating virus was removed, and the cultures were then incubated in the presence of [^{35}S]methionine at 37°C with 5% CO_2 overnight. Cells were harvested and pelleted by centrifugation at 2,000 × *g* for 5 min. The cell pellet was resuspended in cell lysis buffer (0.05 M Tris-HCl [pH 7.5], 0.6 M KCl, 0.5% Triton X-100), incubated at room temperature for 5 min, and centrifuged at 15,000 × *g* for 5 min. Radiolabeled HA was immunoprecipitated with anti-H1 monoclonal antibodies linked to protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden). Immunoprecipitated HA was then washed twice with buffer which did not contain protease inhibitors. Immunoprecipitates were treated with 10 to 30 μl of concentrated bacterial supernatant, based on the relative caseinolytic activity of the bacterial supernatant, and incubated for 60 min at 37°C. For purified *P. aeruginosa* elastase and alkaline protease, immunoprecipitated HA was treated with 10 μl of 20-μg/ml protease. The supernatant was then removed, and the samples were mixed with Laemmli sample buffer containing 5% β-mercaptoethanol, boiled, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Assay of influenza virus activation by bacterial supernatants. SW/WI HG virus was treated with the bacterial supernatants to determine the effect on influenza virus infectivity. Uncleaved SW/WI HG virus was diluted in Tris-saline (0.05 M Tris-HCl [pH 8], 0.9% NaCl) to a titer of 5×10^7 50% egg infective doses (EID_{50}s)/ml. Twenty microliters of diluted virus (10^6 EID_{50}s) was incubated with 10 to 30 μl of concentrated bacterial supernatant at 37°C for 60 min. The volume of concentrated bacterial supernatant used was the same as that for the HA cleavage studies. Virus titers were determined by standard EID_{50} assay (15). This method of determining the effect of proteolysis on viral infectivity was used because it can detect both cleavage activation and inactivation of the treated virus.

Western immunoblot analysis for the detection of *Pseudomonas* elastase and alkaline protease. Preparations of purified elastase, alkaline protease, and concentrated *Pseudomonas* culture supernatants were subjected to electrophoresis on SDS–10% polyacrylamide gels (21). Proteins from the SDS-PAGE gels were electroblotted to nitrocellulose (Trans-Blot; Bio-Rad) with a Multiphor II electrophoresis system (LKB, Broma, Sweden). The membranes were blocked with 2% skim milk in Tris Tween-buffered saline (TTBS; 50 mM Tris [pH 7.4], 0.9% NaCl, 0.1% Tween 20). The membranes were then incubated for 1 h with rabbit antielastase or anti-alkaline protease antisera diluted 1:1,000 in TTBS, followed by a 1-h incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, Inc., San Francisco, Calif.) diluted 1:10,000 in TTBS. The proteins were detected by incubation with 66 μl of nitroblue tetrazolium chloride (Boehringer Mannheim, Indianapolis, Ind.) and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer Mannheim) in 10 ml of substrate buffer (0.1 M Tris, 0.1 M NaCl, 0.005 M $MgCl_2$).

Amino-terminal sequencing of HA2 cleavage fragments. To determine the site of HA cleavage by purified proteases and bacterial supernatants, we isolated and

TABLE 1. Protease-producing bacteria isolated from nasal swabs or nasal washes of healthy pigs

Swine nasal flora isolate	Diam (mm) of clearing zone for isolate from pig[a]										
	1	2	3	4	5	6	7	8	9	10	11
Staphylococcus chromogenes	—[b]	22	—	18	—	—	19	19	—	18	—
Aeromonas caviae	—	—	—	—	—	—	9	11	6	—	—
Staphylococcus hyicus	—	—	—	—	—	—	—	—	11	10	—
Pseudomonas aeruginosa	—	22	—	—	—	—	—	—	—	—	—
Enterococcus sp.	12	—	—	—	—	—	—	—	—	—	—
Stenotrophomonas maltophilia	—	—	—	—	—	—	—	7	—	—	—

[a] Values represent the diameter of the clearing zone on the casein agar assay produced by 10 μl of concentrated bacterial supernatant. Trypsin (10 μg/ml) gives a clearing zone of 15 mm.

[b] —, no proteolytic isolates of the specified bacteria were obtained from the pig sample.

sequenced the amino terminus of the HA2 digestion fragments. H1-specific monoclonal antibodies were covalently linked to Affigel-10 beads (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. Nonradiolabeled infected cell lysates were immunoprecipitated and treated with 10 μl of purified protease or bacterial culture supernatants showing HA cleavage activity. After incubation at 37°C for 60 min, the samples were washed three times with PBS–0.1% Triton X-100. The HA was eluted in two 15-μl washes with 1 M propionic acid (pH 4.0) and neutralized with 3 μl of 1 M sodium phosphate buffer (pH 8). The eluted samples were subjected to tricine SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane (ProBlott; Applied Biosystems, Foster City, Calif.) by the method of Matsudaira (23). The Western blots were stained with Coomassie blue, and the band coinciding with the HA2 fragment was excised for amino-terminal peptide sequencing. Amino-terminal sequencing was performed by the Macromolecular Structure Facility at Michigan State University.

RESULTS

Protease production by swine respiratory bacteria. Secreted proteases were not detected for any of the swine respiratory pathogen isolates. However, several proteolytic bacteria were isolated from the nasal cavities of normal pigs. All of the proteolytic bacteria secreted proteases in TSB, while only some of the proteolytic isolates secreted proteases in BHIB and/or dialyzed BHIB. Protease-secreting bacteria were isolated from 7 of 11 (64%) pig nasal samples (Table 1). Multiple proteolytic bacteria were isolated from five of the pigs. *Staphylococcus chromogenes* was the most frequent protease-secreting bacteria isolated. Supernatants from *S. chromogenes* and *P. aeruginosa* PaSw demonstrated the greatest caseinolytic activity.

All of the caseinolytic supernatants were also protease positive by the PepTag assay (Fig. 1). In addition, all of the supernatants showed digestion fragments similar to those observed after trypsin digestion. This suggested that some of these bacterial supernatants contained proteases that have cleavage site specificity similar to that of trypsin.

Digestion of HA by bacterial supernatants. Radiolabeled HA from infected cell lysates was immunoprecipitated and treated with the bacterial supernatants. Digestion fragments were analyzed by SDS-PAGE to determine the HA cleavage activity. Bacterial supernatants from strains PaSw and PAO1 showed significant HA cleavage activity (Fig. 2). Supernatants from the *Bacillus* sp. also had strong HA cleavage activity (data not shown). Treatment with all three supernatants resulted in two cleavage fragments with migrations similar to those of the HA1 and HA2 digestion fragments observed with trypsin digestion (Fig. 2). The proteolytic supernatants from the remaining nasal flora isolates showed minimal cleavage activity on the HA.

Effect of bacterial supernatants on activation of uncleaved influenza virus. We tested the effect of the concentrated bacterial supernatants on cleavage activation of influenza virus with intact HA. Uncleaved SW/WI HG stock virus was treated in vitro with selected bacterial supernatants and titrated in 10-day-old embryonated chicken eggs. This method allowed us to detect cleavage activation of the virus, as well as inactivation that was possibly due to inappropriate cleavage of the HA. The results were recorded as either an increase or decrease in EID_{50} per milliliter compared to that of virus treated with PBS

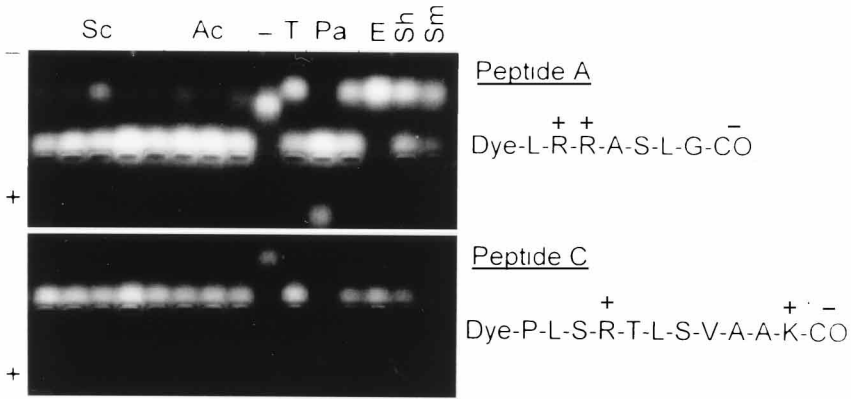

FIG. 1. PepTag protease digestion assay for bacterial supernatants. Dye-linked peptides were treated with the bacterial supernatants according to the manufacturer's (Promega) directions. The samples were loaded in the center wells of a 1% agarose gel and subjected to electrophoresis. Dye-linked digestion fragments migrate toward the anode or cathode based on their net charge and size. This is a sensitive proteolytic assay and also gives some indication of protease site specificity. Sc, *S. chromogenes* (lanes 1 to 5, isolates 22, 35, 3-22A, 4-4, and 4-10A, respectively); Ac, *A. caviae* (lanes 6 to 8, isolates 4-15, 4-18, and 5-12B, respectively); −, negative control (lane 9); T, trypsin at 10 μg/ml (lane 10); Pa, *P. aeruginosa* PAO1 (lane 11) and isolate PaSw (lane 12); E, *Enterococcus* sp. (lane 13, isolate 10); Sh, *S. hyicus* (lane 14, isolate 5-7); Sm, *S. maltophilia* (lane 15, isolate 4-24).

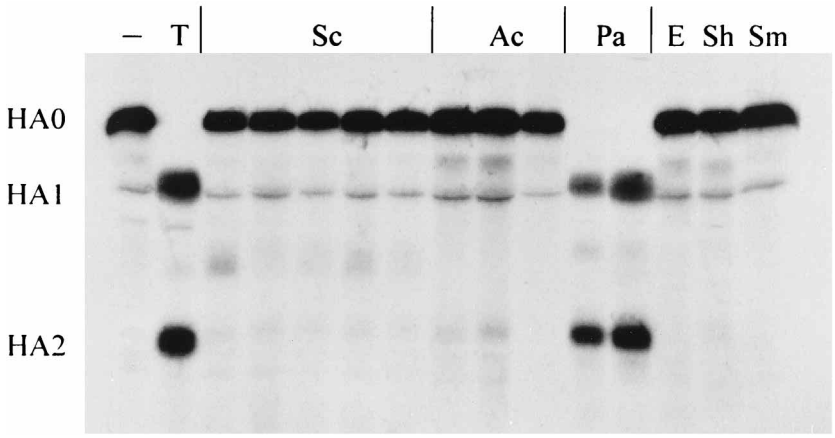

FIG. 2. SDS-PAGE of digestion fragments from immunoprecipitated HA that was treated with bacterial supernatants. MDCK cells were infected with SW/WI HG (H1N1) in the presence of [^{35}S]methionine. HA was immunoprecipitated from cell lysates with H1-specific monoclonal antibodies linked to protein A-Sepharose beads. Immunoprecipitated HA was treated with concentrated TSB culture supernatants or trypsin at 37°C for 60 min. Treated samples were analyzed by SDS-PAGE and autoradiography. –, TSB negative control (lane 1); T, trypsin at 10 μg/ml (lane 2); Sc, *S. chromogenes* (lanes 3 to 7, isolates 22, 35, 3-22A, 4-4, and 4-10A, respectively); Ac, *A. caviae* (lanes 8 to 10, isolates 4-15, 4-18A, and 5-12B, respectively); Pa, *P. aeruginosa* PAO1 (lane 11) and isolate PaSw (lane 12); E, *Enterococcus* sp. (lane 13, isolate 10); Sh, *S. hyicus* (lane 14, isolate 5-7); Sm, *S. maltophilia* (lane 15, isolate 4-24).

(Fig. 3). Both trypsin (10 μg/ml) and *Bacillus* sp. culture supernatant resulted in nearly a 100-fold increase in virus infectivity titer. However, all of the swine respiratory bacterial supernatants resulted in some decrease in virus infectivity titer. Culture supernatants from PaSw, *S. hyicus*, and the laboratory PAO1 strain decreased the virus titer by nearly 100-fold. Since the *P. aeruginosa* isolates also showed strong HA cleavage activity, we investigated which *Pseudomonas* proteases were responsible for the HA cleavage and whether they also accounted for the decrease in virus titer.

Investigation of *P. aeruginosa* proteases and cleavage of influenza virus HA. Supernatants from the PAO1 and swine nasal isolate of *P. aeruginosa* (PaSw) were compared with purified *Pseudomonas* elastase and alkaline protease. First, we compared the HA digestion activities of these proteases (Fig. 4A). *Pseudomonas* elastase showed strong HA cleavage activity, resulting in digestion fragments indistinguishable from

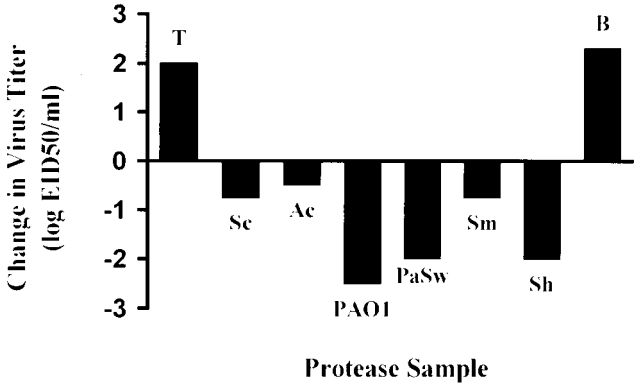

FIG. 3. Effect of bacterial supernatants with protease activity on influenza virus infectivity. Virus with uncleaved HA (SW/WI HG) was treated with bacterial culture supernatants or trypsin. The titer of infectious virus was determined by inoculation of 10-day-old embryonated chicken eggs with 100 μl of treated virus, in triplicate, at $\log_{10}$ dilutions. Infected eggs were identified by HA activity of the allantoic fluid 60 h postinfection, and the EID_{50} was determined. Each bar represents the change in virus titer compared to the titer of virus treated with PBS alone. T, trypsin at 10 μg/ml; Sc, *S. chromogenes* 4-4; Ac, *A. caviae* 4-18; Sm, *S. maltophilia* 4-24; Sh, *S. hyicus* 5-7; B, *Bacillus* sp. isolate 37.

those observed with the culture supernatants and trypsin. In contrast, *Pseudomonas* alkaline protease did not show significant digestion of the HA. This suggested that elastase was the protease responsible for the effect we were seeing with the culture supernatants.

To provide further evidence for this, we performed Western blotting analysis of the samples and immunostained them with antielastase and anti-alkaline protease antibodies (Fig. 4B and C). We were unable to detect alkaline protease in the culture supernatants for both the PAO1 and PaSw isolates. However, TSB supernatants of both isolates showed protein bands that reacted with the antielastase antibody. The PAO1 strain produced the expected 33-kDa protein that corresponds to purified elastase. In contrast, antielastase antibody reacted with a 55-kDa protein from PaSw. This may be an alternative form of elastase or a different protein that cross-reacts with the antielastase antibody.

Finally, we also tested the effect of purified elastase on uncleaved virus and found that it also decreased the virus titer by nearly 100-fold. These data demonstrate that *Pseudomonas* elastase cleaves the HA and decreases viral infectivity, possibly by digesting the HA at a point just upstream or downstream from the normal cleavage activation site.

HA cleavage site specificity of elastase and the bacterial culture supernatants. To determine the specific HA cleavage site for the proteases, amino-terminal sequencing of purified HA2 digestion fragments was performed. Digestion of the HA with purified elastase or the TSB culture supernatants from the PAO1 and PaSw isolates resulted in an amino-terminal sequence of (*N*)-L-F-G-A-I. This sequence corresponds to cleavage of the HA just one amino acid downstream from the normal trypsin cleavage site. In contrast, digestion with the *Bacillus* sp. supernatant resulted in an amino-terminal sequence of (*N*)-G-L-F-G-A, which coincides exactly with the HA2 amino-terminal sequence predicted for trypsin digestion of the HA.

DISCUSSION

Swine influenza viruses isolated from field outbreaks frequently induce only mild disease in experimentally infected pigs. Differences in severity of infection have been attributed

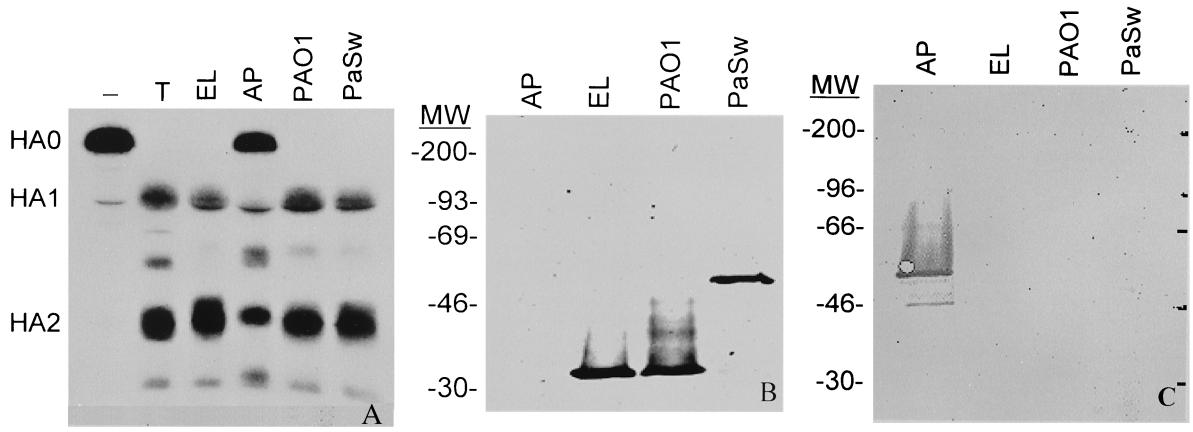

FIG. 4. Comparison of *P. aeruginosa* bacterial supernatants with purified *Pseudomonas* elastase (EL) and alkaline protease (AP). (A) Digestion of immunoprecipitated HA by proteases. –, PBS; T, trypsin. (B) Western blot analysis of purified proteases and culture supernatants immunostained with antielastase antibody. (C) Western blot analysis of purified proteases and culture supernatants immunostained with anti-alkaline protease antibody. MW, molecular mass (kilodaltons).

to both environmental conditions and microbial coinfection. Recent interest has focused on the role of microbial proteases in potentiating cleavage activation of influenza viruses in the respiratory tract. At least four possible mechanisms have been proposed, including (i) direct cleavage of the HA; (ii) activation of host proteases capable of cleaving the HA, such as plasmin, thrombin, or kallikrein; (iii) destruction of host protease inhibitors; and (iv) induction of host inflammatory response causing increased leakage of host proteases (1, 30). Potentiation of HA cleavage by microbial proteases could contribute to disease severity by increasing virus titers, spread, and anatomic distribution in the respiratory tract.

There is limited information concerning the occurrence of protease-secreting bacteria in the respiratory tract of humans and domestic animals. In the present study, we examined clinical isolates of pathogenic respiratory bacteria and nasal flora of pigs for protease activity. In many instances, swine influenza is complicated by additional bacterial pathogens. This is particularly true with *H. parasuis*, which can produce a severe respiratory disease syndrome during influenza virus coinfection (31, 36). Thus, we speculated that these organisms might produce proteases that potentiate cleavage activation of influenza virus. Using a variety of different culture conditions, we did not detect secreted proteases from any of the bacterial pathogens. Our findings suggest that direct cleavage of influenza virus HA is not a mechanism by which these pathogenic bacteria contribute to influenza virus pathogenesis.

Since influenza is typically an upper respiratory infection in pigs, we also examined normal nasal flora for protease production. Under a variety of culture conditions, we isolated several proteolytic bacteria. There was considerable variation in the number of protease-producing isolates and the degree of proteolytic activity between pigs (Table 1). *S. chromogenes* was the proteolytic bacterium most frequently isolated, and these culture supernatants showed the highest degree of caseinolytic activity. *S. chromogenes*, previously classified as *S. hyicus* subsp. *chromogenes*, is a common resident on the skin of pigs and is not considered a pathogen of the swine upper respiratory tract (36).

The remaining proteolytic bacteria were isolated less frequently. *A. caviae* is isolated from environmental sources such as soil, water, and marine-based by-products. It is not considered a pathogen of swine, although it may infect wounds and cause gastrointestinal disease in humans. However, it is an opportunistic commensal bacterium, and isolation is probably a reflection of environmental exposure. *S. hyicus* is a swine pathogen causing exudative epidermitis (36). The organism is carried on the skin of asymptomatic animals. Proteolytic isolates have previously been recovered from the upper respiratory tracts of poultry (5). The *P. aeruginosa*, *Enterococcus* sp., and *Stenotrophomonas maltophilia* isolates are most likely opportunistic invaders from environmental sources.

It is interesting that no proteolytic bacteria were isolated from 4 of the 11 pigs. This suggests that the proteolytic environment of the nasal mucosa may differ between animals. If microbial proteases play a role in potentiation of influenza virus infection, this variability could account for some of the differences in disease severity. Variation in the number of proteolytic isolates and in the degree of proteolytic activity has also been observed in the upper respiratory tracts of poultry (5).

S. aureus produces several proteases and can be isolated from the upper respiratory tracts of poultry, horses, and humans (5, 7, 29). We did not detect proteolytic *S. aureus* from any of the pigs sampled in this study. This is of interest, since some *S. aureus* isolates secrete proteases that directly cleave and activate influenza virus HA (35). Our findings suggest that *S. aureus* is unlikely to play a significant role in direct cleavage activation of influenza virus in healthy pigs under natural conditions. One alternative possibility is that initial infection with influenza virus alters the mucosal environment and allows increased colonization of additional proteolytic bacteria. Increased colonization of *S. aureus* after influenza virus infection is reported in horses and humans (29, 38). Examination of the proteolytic nasal flora following influenza virus infection would be interesting in this regard.

All of the bacterial supernatants that showed caseinolytic activity were also positive on the PepTag assay and produced digestion fragments similar to those observed with trypsin (Fig. 2). Trypsin is the prototypical serine protease used for cleavage activation of influenza virus in vitro, cleaving HA0 on the carboxyl side of a specific arginine residue (20, 22, 27). This observation led us to believe that some of the bacterial proteases might contribute to cleavage activation by direct cleavage of the influenza virus HA. However, only supernatants from *P. aeruginosa* (Fig. 2) and the *Bacillus* sp. isolate (data not shown) demonstrated significant digestion of HA. In both

cases, the size of the digestion fragments indicated cleavage at a site either identical to or very near the trypsin cleavage site.

We utilized an egg infection assay to determine the effect of the bacterial supernatants on viral infectivity for a variety of reasons. The allantoic fluid of eggs contains a protease that activates influenza virus and allows replication in embryonated chicken eggs (9). When eggs are inoculated with uncleaved virus, the allantoic fluid proteases cleave some of these viruses sufficiently to allow infection of the allantoic cells. Pretreatment with trypsin increases the virus titer above this basal level. Alternatively, inappropriate cleavage of the HA by other proteases may result in decreased virus titers. This assay system allowed us to concurrently test for both viral activation and inactivation by proteases. In preparing the uncleaved virus stock, it is critical that the initial inoculum is completely removed. Otherwise, residual cleaved virus will contaminate the newly produced uncleaved virus and viral inactivation may not be observed. We found that repeated washes did not sufficiently remove residual virus. Instead, neutralization of the inoculum by acid treatment was required (35).

We also attempted similar assays with various tissue culture systems in order to eliminate the potential confounding effects of proteases in the allantoic fluid of eggs. However, the bacterial supernatants were invariably toxic to the cells used (MDCK cells and chicken embryo fibroblasts). Thus, the cell culture system could not be utilized without purification of the proteases from each bacterial isolate. In addition, even the purified *Pseudomonas* elastase was toxic to the cells at the concentrations required to provide HA cleavage. Because of these difficulties, we found the egg assay method to be more sensitive and reproducible than tissue culture assays, such as the modified plaque assay described previously (35, 42).

Both trypsin and the laboratory *Bacillus* sp. isolate increased viral infectivity. The results with the *Bacillus* sp. isolate demonstrate that our methods are capable of identifying bacteria that produce proteases capable of direct cleavage activation of influenza virus. However, none of the swine bacterial supernatants tested were able to increase viral infectivity. Rather, we saw a 100-fold decrease in virus infectivity after treatment with the swine *P. aeruginosa* and *S. hyicus* isolates. We are unsure of the reason for the decreased infectivity after treatment with the *S. hyicus* supernatant, since this isolate did not show significant HA proteolytic activity. It is possible that other secreted bacterial proteins inhibited the virus.

Both the swine and laboratory *P. aeruginosa* isolates cleaved the HA and decreased viral infectivity. *P. aeruginosa* produces several different proteases, the most common being elastase and alkaline protease (24). A previous study determined that a protease from *P. aeruginosa* was unable to cleave the influenza virus HA (30). Unfortunately, it is unclear which *P. aeruginosa* protease was studied. However, the *Pseudomonas* protease did enhance virus titers and pathogenicity in mice, probably by one of the alternative mechanisms described above.

In the present study, we demonstrate that *Pseudomonas* elastase cleaves the HA one amino acid downstream from the normal trypsin cleavage site. This results in the loss of the amino-terminal glycine from the fusion domain on HA2. The cleavage site specificity of *Pseudomonas* elastase is on the imino side of bulky hydrophobic amino acids, such as leucine and phenylalanine (24). The cleavage site sequence of the HA for Sw/WI/1915/83 is S_{340}-I-Q-S-R*G-L-F-G-A-I_{350} (* marks the site of trypsin cleavage). Thus, the cleavage point observed for *Pseudomonas* elastase is in agreement with the site specificity on the basis of the N-terminal sequence of the HA2 digestion fragment, (*N*)-L-F-G-A-I.

Analysis of fusion peptide mutants of influenza virus has demonstrated the importance of the amino-terminal residue in fusion activity (8, 33). *Pseudomonas* elastase is a metalloprotease similar to the *Bacillus thermoproteolyticus* protease, thermolysin (EC 3.4.24.27). Cleavage of HA0 by thermolysin also results in an HA2 fragment that lacks the amino-terminal glycine, resulting in the loss of fusion activity (33). Loss of fusion activity due to inappropriate cleavage of HA0 is the most likely explanation for the decreased infectivity observed after treatment with *P. aeruginosa* supernatants or purified *Pseudomonas* elastase.

Previous studies have demonstrated that proteases from *S. aureus* and *A. viridans* are capable of direct cleavage activation of some influenza viruses (30, 34, 35). In both cases, disease severity was increased after coinfection with bacteria and virus in pneumonia models. Virus titers in lung tissue of coinfected mice were increased 100- to 1,000-fold over virus infection alone (30, 34, 35). These studies led to speculation that bacterial proteases play a direct role in cleavage activation of influenza virus in natural infection. While we were able to demonstrate the presence of proteolytic bacteria in the nasal flora of pigs, none of the bacteria secreted proteases capable of activating a common swine influenza virus in vitro.

In this study, we used a variety of culture media to increase our likelihood of detecting proteolytic aerobic bacteria. While differences between culture media were observed, all of the proteolytic bacteria identified secreted proteases in TSB, indicating this is a good medium for screening and detecting proteolytic bacteria. However, bacterial growth and protease secretion in the respiratory tract may not be comparable to those observed under the in vitro conditions used in this study. It is possible that the environmental conditions of the respiratory mucosa affect both the amount and type of bacterial protease secreted. Attempts at direct recovery of bacterial proteases from the respiratory tract would need to be performed to evaluate this possibility. In addition, obligate anaerobic flora were not examined in this study and could be another source of proteases capable of influenza virus cleavage activation.

Microbial proteases can potentiate cleavage activation of influenza virus by mechanisms other than direct cleavage of the HA. Studies with staphylokinase, streptokinase, and *S. marcescens* protease indicate that they potentiate cleavage activation by activating host proteases such as kallikrein, plasmin, or thrombin (1, 30). We did not test the proteolytic bacteria isolated in this study for their ability to activate host proteases. In addition, it is possible that microbial proteases could contribute to host protease activity by increasing inflammation or destroying endogenous protease inhibitors.

It is still unknown if microbial proteases provide a necessary contribution to influenza virus infection in the respiratory tracts of natural hosts such as pigs, horses, or humans. In the present study, we isolated several aerobic bacteria from the respiratory tracts of pigs that secrete proteases in vitro. However, none of these isolates resulted in direct cleavage activation of influenza virus, and one isolate, *P. aeruginosa*, actually decreased influenza virus infectivity due to cleavage at an aberrant site. These results suggest that direct cleavage activation of influenza virus HA by aerobic proteolytic bacteria is not a common event in the normal swine respiratory tract. Further research needs to be performed to determine the relative importance of host and microbial proteases in cleavage activation of influenza virus.

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