Serine Protease PrtA from Streptococcus pneumoniae Plays a Role in the Killing of S. pneumoniae by Apolactoferrin

Shaper Mirza,1,6 Landon Wilson,2,5 William H. Benjamin, Jr.,3,1 Jan Novak,1 Stephen Barnes,2,5 Susan K. Hollingshead,1 and David E. Briles1,4,*

Departments of Microbiology,1 Pharmacology and Toxicology,2 Pathology,3 and Pediatrics,4 Division of Epidemiology, and Targeted Metabolomics and Proteomics Laboratory,5 University of Alabama at Birmingham, Birmingham, Alabama 35294, and University of Texas Health Science Center, School of Public Health, Houston, Texas6

Received 10 May 2010/Returned for modification 7 June 2010/Accepted 10 March 2011

It is known that apolactoferrin, the iron-free form of human lactoferrin, can kill many species of bacteria, including Streptococcus pneumoniae. Lactoferricin, an N-terminal peptide of apolactoferrin, and fragments of it are even more bactericidal than apolactoferrin. In this study we found that apolactoferrin must be cleaved by a serine protease in order for it to kill pneumococci. The serine protease inhibitors were able to block killing by apolactoferrin but did not block killing by a lactoferrin-derived peptide. Thus, the killing of pneumococci by apolactoferrin appears to require a protease to release a lactoferricin-like peptide(s). Incubation of apolactoferrin with growing pneumococci resulted in a 12-kDa reduction in its molecular mass, of which about 7 to 8 kDa of the reduction was protease dependent. Capsular type 2 and 19F strains with mutations in the gene encoding the major cell wall-associated serine protease, prtA, lost much of their ability to degrade apolactoferrin and were relatively resistant to killing by apolactoferrin (P < 0.001). Recombinant PrtA was also able to cleave apolactoferrin, reducing its mass by about 8 kDa, and greatly enhance the killing activity of the solution containing the apolactoferrin and its cleavage products. Mass spectroscopy revealed that PrtA makes a major cut between amino acids 78 and 79 of human lactoferrin, removing the N-terminal end of the molecule (about 8.6 kDa). The simplest interpretation of these data is that the mechanism by which apolactoferrin kills Streptococcus pneumoniae requires the release of a lactoferricin-like peptide(s) and that it is this peptide(s), and not the intact apolactoferrin, which kills pneumococci.

Streptococcus pneumoniae is a major inhabitant of the human upper respiratory tract and can cause pneumonia, sepsis, meningitis, and otitis media (21a). Pneumococci are common inhabitants of the nasopharynx of healthy children and adults. Pneumococci can cause serious disease when they leave the nasopharynx and successfully invade the middle ear, lungs, blood, or brain (21a). Although specific antibody can mediate protection against pneumococcal infection (14, 53), there is also evidence that antibody is not necessary for immunity to colonization and that innate immune plays a significant role (30, 50, 58). Moreover, the molecular mechanisms involved in the interaction of S. pneumoniae with the innate immune system are incompletely understood.

Known innate immune system components that protect against bacterial infection in the upper airways include mucus flow, antibacterial proteins, such as lysozyme and lactoferrin, and antibacterial peptides, such as α-defensins and β-defensins (57). Human lactoferrin (hLf) has been reported to play a number of different roles in mammals, including immunoregulation, iron sequestration, promotion of calcium uptake, and bactericidal activity (52). The observation that pneumococcal surface protein A (PspA) specifically inhibits in vitro killing of pneumococci by apolactoferrin (apo-hLf), the iron-free form of lactoferrin, suggests that one of the functions of PspA may be to protect pneumococci from killing by apo-hLf in vivo. It has been previously reported that apo-hLf but not the iron-bound, or holo human, lactoferrin (holo-hLf) can kill pneumococci and many other bacteria in vitro (5, 6, 13). Bacteria can also be killed by several tryptophan-containing cationic peptides that can be cleaved from among the first 100 amino acids at the N-terminal end of apo-hLf by the action of several different proteases and by extended incubation under mildly acidic conditions (17, 32, 36, 39, 51). These bactericidal peptides are collectively termed lactoferricin-like peptides (LLP). Synthetic versions of two of these peptides have been shown to kill pneumococci (31). LLP are 10 to 100 times more bactericidal on a weight basis than is apo-hLf (24, 34, 54). Like many other bactericidal cationic peptides, LLP are thought to exert antimicrobial activities through the disruption of membranes (33, 44). This action of LLP can be explained by the amphipathic structure of these cationic peptides, which allows them to bind and disrupt the integrity of negatively charged bacterial membranes (36). These properties of LLP raise the possibility that killing by apo-hLf is dependent on cleavage of one or more LLP from the apo-hLf molecule. This hypothesis is supported by our finding, reported in this article, that the killing of S. pneumoniae by apo-hLf can be blocked by serine protease inhibitors.

Human apolactoferrin has an inefficient yet highly specific protease activity (25, 38), but there is no evidence that it catalyzes autocleavage of apo-hLf or that one apo-hLf molecule can cleave another apo-hLf molecule. If LLP were released from apo-hLf by host proteases in serum and secretions,
TABLE 1. Wild-type pneumococcal strains and their isogenic prtA-negative mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Capsule type</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>2</td>
<td>Wild-type prtA*</td>
<td>7</td>
</tr>
<tr>
<td>EF3030</td>
<td>19F</td>
<td>Wild-type prtA*</td>
<td>3</td>
</tr>
<tr>
<td>MJ04.1</td>
<td>19F</td>
<td>prtA mutant, Emr'</td>
<td>This study</td>
</tr>
<tr>
<td>MJ04.2</td>
<td>2</td>
<td>prtA mutant, Emr'</td>
<td>This study</td>
</tr>
<tr>
<td>MJ04.11</td>
<td>19F</td>
<td>prtA mutant/prpA mutant, Emr/Tef'</td>
<td>This study</td>
</tr>
</tbody>
</table>

TABLE 2. Sequence of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>5-GAC GAC GAC AAG ATG AAA AAA AGC</td>
</tr>
<tr>
<td>M13R</td>
<td>5-TTC TTTATA GAG TTC TTT GTC TGT-3</td>
</tr>
<tr>
<td>PrtAF</td>
<td>5-GAC GAC GAC ATG AAA AAA AGC</td>
</tr>
<tr>
<td>PrtAR</td>
<td>5-TTC TTATATA GAG TTC TTT GTC TGT-3</td>
</tr>
<tr>
<td>PrtAsense</td>
<td>5-GAC GAC GAC ATG AAA AAA AGC</td>
</tr>
</tbody>
</table>

the highly charged peptides might be lost by associating with host molecules before they encounter bacteria. It seemed more likely that apo-hLf relies on proteases at the bacterial surface to release LLP. This would ensure that the LLP was released only in close proximity to bacterial cells. Since PrtA is thought to be a major surface serine protease of pneumococci (11, 59), we have evaluated the ability of PrtA to cleave apo-hLf and to facilitate the killing of pneumococci by apo-hLf.

PrtA is a cell-wall-associated serine protease that is produced as a full-length 240-kDa precursor, which is processed to yield a mature 215-kDa protein. PrtA has been reported to be an important virulence factor in a mouse peritoneal challenge model (11), but its role in colonization and invasion is not known. The prtA gene is present in almost all pneumococci and appears to be highly conserved except for the regions between nucleotides 100 and 300 and between nucleotides 4201 and 4300 (11). Our present studies show that a functional prtA gene is required for much of the proteolytic activity of pneumococci against apo-hLf. The action of PrtA on apo-hLf resulted in a reduction in mass of apo-hLf concomitant with an increase in the bactericidal activity of the apo-hLf-containing solution. Moreover, the expression of PrtA by pneumococci was required for maximal susceptibility to killing by apo-hLf.

MATERIALS AND METHODS

Materials. Lactoferrin used in this study was obtained from human milk. Human lactoferrin (iron free) and human hololactoferrin (iron saturated) were purchased from Sigma (St. Louis, MO). The N-terminal 11 amino acids of the human LLP, LFNI (31), was synthesized by Celtec Peptide Synthesis (Nashville, TN). The sequence of the peptide is Gly-Arg-Arg-Arg-Arg Ser-Val-Gln.

Bacterial growth conditions. Strains of S. pneumoniae (Table 1) were maintained as frozen stocks in Todd-Hewitt broth with 5% yeast extract (THY) (Becton Todd Hewitt broth; Becton, Dickinson and Company, Franklin Lakes, NJ) at ~80°C and then grown in THY broth until an optical density (OD) of 0.5 was achieved. Cultures were then diluted in fresh THY to an optical density of 0.5 (OD600). The cultures were then diluted in blood agar plates containing 0.3 µg/ml erythromycin. The final mutant strains were MJ04.1 (a PrtA-negative strain of EF3030 mutant) and MJ04.2 (a PrtA-negative D39 mutant). The integration of the plasmid in the correct double mutants were generated by transformation of the prtA-deficient strains.

Construction of prtA-deficient strains. The mutation was constructed by insertional inactivation (55) of the prtA gene in strains EF3030 and D39. A construct containing a 700-bp internal fragment of the prtA gene (nucleotides 591 to 1291) was cloned into the multiple cloning site of plasmid pJD9. This construct (11) was a kind gift from Gregor Zysk, Department of Medical Microbiology and Virology, University of Dusseldorf, Dusseldorf, Germany. The TOP10 Escherichia coli cells (Invitrogen, Carlsbad, CA) were transformed with the plasmid for further propagation, followed by isolation of plasmid DNA. Capsule type 19F strain EF3030 and capsule type 2 strain D39 were transformed with this construct using standard techniques, modified from those of Youther et al. (56). Briefly, strains to be transformed were grown in THY to an optical density at 600 nm (OD600) of 0.5 and diluted 1:50 in competence medium (CTM) (THY with 0.2% bovine serum albumin, 0.2% glucose, and 0.02% CaCl2) with 500 ng/ml (each) competence-stimulating peptides CSP1 and CSP2 (23) to induce competence and finally the addition of plasmid DNA. Bacteria were incubated at 37°C for 2 h, followed by plating on blood agar plates containing 0.3 µg/ml erythromycin. The final mutant strains were MJ04.1 (a prtA-deficient EF3030 mutant) and MJ04.2 (a prtA-deficient D39 mutant). The integration of the plasmid in the correct position was determined by PCR using a forward primer specific for the prtA (SP1) gene and a reverse primer homologous to the multiple cloning site in the plasmid (M13 reverse) (Table 2). The presence of a 500-bp amplified product in the prtA mutant strains and its absence in the wild-type strains confirmed the insertion of the plasmid and thus the disruption of the prtA gene in the pneumococcal chromosome.

The pop4 prtA double mutants were generated by transformation of the pop4-negative strain of EF3030 (10) (TRE 141) with plasmid pSF143, containing the insert for prtA. The resulting strain was named MJ04.11. Briefly, prtA was amplified using the primers PrtAR, which binds at nucleotide 2194, and PrtAF, which binds at nucleotide 1429, which amplified a 765-bp internal fragment of the prtA. The 765-bp fragment was then ligated into a TOPO vector (TOPO PCR II) (Invitrogen Corporation, Carlsbad, CA). The construct, which was named pop4-prtA-TOPO, was digested using the restriction enzyme EcoRI. The digested fragment was then cloned into the EcoRI site of pSF-143 (47), which contains a tetracycline resistance marker. The construct was transformed into TOP10 E. coli cells (Invitrogen). Purified plasmid from E. coli was then used to transform pneumococcal strain EF3030. Transformants were selected on tetracycline and erythromycin plates.

Treatment of S. pneumoniae strains with human apolactoferrin. The comparative killing rates of prtA strains relative to that of the parent strain were determined by incubating bacteria in assay saline (AS) consisting of 150 mM NaCl, 1 mM MgCl2, and 50 µM CaCl2 with 3.1 µM apo-hLf, as described previously (31). Pneumococcal cultures were grown as described previously to an optical density of 0.5 (A590). The cultures were then diluted in fresh THY to an
optical density of 0.001 to 0.01 and grown again until the optical density reached 0.15 (31). This procedure was necessary to ensure that the bulk of bacterial cells in each assay were actively dividing. Bacterial cells were washed once with AS, resuspended in AS with or without 3.1 μM apo-hLf, and incubated at 37°C for 1 h. Viability was determined by plating the cells on blood agar plates or, for strains containing mutants carrying an erythromycin-resistance cassette, on blood plates with 0.3 μg/ml erythromycin. Log killing was calculated from a comparison of the CFU counts for the treated samples with the CFU counts of untreated controls that had been similarly incubated but in the absence of apo-hLf (31). It should be noted that since these incubations were carried out in AS, which lacks nutrients, very little if any growth or death (<5%) was seen in the control groups compared to CFU at time zero.

Assay for the ability of protease activity in live S. pneumoniae to degrade apo-hLf. S. pneumoniae strains D39 and EF3030 and their isogenic prtA mutants were grown in either THY or C-V medium and treated with lactoferrin. To determine if the activity of the protease is growth phase dependent, aliquots were removed from growing cultures at optical densities (A595) of 0.2, 0.3, 0.4, 0.5, and 0.7. Each aliquot was immediately treated with 0.62 μM apo-hLf for 1 h. The concentration of 0.62 μM apo-hLf was chosen since little killing of bacteria was observed previously at this concentration after 1 h incubation at 37°C (31).

To help control for potential effects of growth conditions and nutritional status on the expression of PrtA, bacteria were treated with either THY or C-V medium prior to treatment with apo-hLf. The effect of divalent ions on the activity of PrtA to cleave lactoferrin was determined by adding different concentrations of CaCl2 (1 mM and 5 mM) to the mixture of bacteria and apo-hLf. To determine the optimum time required for the proteolytic cleavage of lactoferrin, reaction mixtures containing bacteria and lactoferrin were incubated for 10, 30, 60, 90, 120, or 240 min, at which time the reactions were stopped by the addition of 7.5 mM PMSF.

To assess the degradation of apo-hLf proteins were separated on a 4 to 12% Bis-Tris-polyacrylamide gradient gel (Invitrogen). Gels were run according to the manufacturer’s instructions, followed by Western blotting. Briefly, 10 μg of total protein was loaded onto the gel and run under denaturing conditions in a 4-morpholine sulonic acid (MES)-SDS buffer system (Invitrogen) until the 27-kDa band of the molecular mass marker ran off the gel. The proteins were then transferred to nitrocellulose using a semidyblotter (Invitrogen) for 20 min at 15 V. The membrane was blocked in 1% bovine serum albumin (BSA) in 1× phosphate-buffered saline (PBS) for 1 h at 37°C. The blots were washed 3 times (5 min each) with PBS containing 0.05% Tween 20 (PBS-T) and were then incubated with monoclonal anti-human lactoferrin for 1 h at 37°C. The primary antibody was diluted 1:1,000 using PBS with 0.1% BSA. Washes were repeated, and biotin-labeled secondary antibody was then added at a final concentration of 1:1,000 (Sigma, St. Louis, MO) mixed with a 1:2,000 dilution of streptavidin-conjugated alkaline phosphatase (Southern Biotechnology Associates Inc., Birmingham, AL). The blots were incubated with the secondary antibody and streptavidin for 45 min, washed, and developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium salt (NBT) (Sigma-Fast tablets; Sigma). The amplified product was then treated with T4 polymerase (Novagen) (plus dNTPs), side triphosphates (dNTPs) and salts from the reaction mixture. The purified sequence 5′AAG ATX, where X is the first nucleotide of the insert-specific primer. Similarly, the reverse primer was designed with 15 bp at the 5′ end with the nucleotide sequence 5′GAG GAG AAG CCC GGT. The amplified product was then treated with T4 polynucleotide kinase (Novagen) (plus dNTPs), ligated into the pET-41 EK/LIC vector by incubation at room temperature for 5 min, and transformed into competent Nova Blue GigaSingles cells (Novagen). Single colonies were selected on kanamycin plates (50 μg/ml) and plasmid DNA was isolated and screened for size by electrophoresis on agarose gels. Transformants containing a product of the correct insert size were selected and grown overnight for plasmid isolation. The presence of the correct insert was confirmed by PCR amplification of the insert from the plasmid. Purified plasmid with the prtA insert was then transformed into BL21(DE3) (Novagen) cells. E. coli containing this plasmid construct was induced using 1 mM isopropyl-β-D-thiogalac-
topyranoside ( IPTG) (Sigma) for protein expression. The recombinant protein, which has both NH2- and COOH-terminal His tags, was then purified using a Talon metal affinity column (Clontech [a Takara Bio company], Mountain View, CA). Identification of protein as recombinant PrtA (PrtA) was carried out by mass spectrometry, using material excised from a Coomassie blue-stained gel.

Examination of effects of PrtA on apo-hLf. To see if PrtA could degrade apo-hLf, 10 μg/ml PrtA was incubated with 10 μg/ml of apo-hLf in a 50 mM Tris-HCl (pH 7.5) and 50 mM CaCl2 reaction mixture for 1 h. Samples were removed at the indicated time points, run on a 10% Bis-Tris gel (Invitrogen), and then transferred to nitrocellulose. Cleavage of apo-hLf was determined by Western blots, which were developed using polyclonal rabbit serum to apo-hLf (Sigma, St. Louis, MO).

To determine if the addition of PrtA would result in a recovery of the activity of apo-hLf to kill prtA mutant pneumococci, a bactericidal activity assay was carried out with 3.1 μM apo-hLf in the presence or absence of 50 μg/ml of PrtA. As a control, pneumococci were also incubated with PrtA alone for 1 h in AS. Killing was determined by plating the bacteria and counting CFU.

To see if degraded lactoferrin is able to kill pneumococci more efficiently than apo-hLf and as efficiently as LLP, 100 μg of apo-hLf was incubated with 50 μg/ml of PrtA for various lengths of time (0.6, 1, and 16 h) in a total of 100 μl of final reaction volume. Pneumococcal strain EFS308 was then incubated with either apo-hLf or with LLP, PrtA or with untreated apo-hLf in AS followed by plating on blood agar plates to determine the number of CFU. In a similar experiment, prtA mutant EFS3030 was also incubated with apo-hLf treated with recombinant PrtA for 16 h under identical conditions.

Determination of cleavage site of PrtA in apolactoferrin. (i) Digestion of lactoferrin by PrtA. Human apolactoferrin at a concentration of 10 μg/ml of was incubated with 50 μg/ml of PrtA for 2 h in 50 mM phosphate buffer with 0.05 mM CaCl2. Protein samples were then prepared for nano-liquid chromatography (LC)-tandem mass spectrometry (LC/MS) as follows.

(ii) In-solution digestion. All protein samples (between 10 and 30 μg) were evaporated in vacuo (SpeedVac) and resuspended in 20 μl of 100 mM Tris-HCl–6 M urea at pH 7.8. Samples were reduced with 2 μl of 200 mM dithiothreitol (DTT) for 60 min at 50°C and then alkylated with 10 μl of 200 mM iodoacetamide at 25°C in the dark for 60 min. Excess iodoacetamide was removed with an additional 10 μl of 200 mM DTT at 25°C for 60 min. The urea concentration was reduced to a final concentration of ~0.6 M by adding 155 μl of Milli-Q water and 5 μl of trypsin digestion solution (20 to 200 ng/ml) dissolved in 100 mM Tris-HCl at pH 7.8. Digestion was carried out at 37°C for a minimum of 16 h in a water bath/incubator. Trypsin activation was halted by adding small increments of glacial acetic or formic acid until samples achieved a pH of ~6. Samples were directly analyzed by LC/MS or desalted with ZipTip C18 disposable columns prior to analysis.

(iii) Nano-LC-tandem mass spectrometry. An aliquot (5 to 10 μl) of each digest was loaded onto a 5-nm-by-100-μm-inside-diameter (i.d.) C18 reverse-phase cartridge at 2 μl/min using a Pal robot (Leap Technologies, Carrboro, NC). After washing of the cartridge for 5 min with 0.1% formic acid in doubledistilled water (dH2O), the bound peptides were flushed onto a 22-μm-by-100-μm reverse-phase column (analytical) and eluted with 5% acetonitrile in 0.1% formic acid at 500 nl/min using a Shimadzu nanopump (Shimadzu, Columbia, MD). The column was washed with 90% acetonitrile–0.1% formic acid for 15 min and then reequilibrated with 5% acetonitrile–0.1% formic acid for 24 min. The eluted peptides were passed directly from the tip into a modified MicrolonSpray interface of an Applied Biosystems MDS-Sciex (Concorde, Ontario, Canada) 4000 Qtrap mass spectrometer. The interface had been rebuilt in order to apply the electrospray voltage through a liquid-liquid junction at the top of the column rather than at the end of the column. This arrangement resulted in very high chromatographic resolution by elimination of the postcolumn dead volume. The IonSpray voltage was 2,500 V, and the declustering potential was 60 V. Ion spray and curtain gases were set at 10 lb/in2 and 15 lb/in2, respectively. The interface-heater temperature was 160°C. Eluted peptides were subjected to a survey MS scan to determine the top two most intense ions. A second scan (the enhanced-resolution scan) determined the charge state of the selected ions. Finally, enhanced product ion scans were carried out to obtain the tandem mass spectrum of the selected parent ions (with the declustering potential raised to 100 V) over the range from m/z 400 to 1,500. Spectra centroids were determined, and spectra were deisotoped by the Analyst software program, version 1.42 (Applied Biosystems). These tandem mass spectrometry data were processed to provide protein identifications using an in-house MASCOT search engine (Matrix Science Inc., Boston, MA) using the human (mammalian) NCBI-nr protein database and one missed protease cleavage site under semimptryic conditions. Variable modifications were allowed for oxidized methionines and fixed modifications for carbamidomethylated cy-
that the protease activity required for killing originated from the bacteria.

**RESULTS**

Effect of protease inhibitors PMSF and SBTI on killing of *S. pneumoniae* by apo-hLf. The previous observation that the lactoferricin and LLP from the N terminus of lactoferrin are a couple of orders of magnitude more bactericidal on a molar basis than apo-hLf (31, 49, 54) suggests that the protease activity may be required to cleave LLP from apo-hLf to facilitate the killing of bacteria. These possibilities were assessed by incubating pneumococci with apo-hLf in the presence of the protease inhibitors PMSF and SBTI. We observed that both of these serine protease inhibitors completely inhibited the bactericidal activity of apo-hLf (Fig. 1A). The required protease activity could have been provided by either lactoferrin (25, 38) or pneumococci (11). Pretreatment of apo-hLf with PMSF, SBTI, or PMSF plus SBTI (followed by inactivation of the excess protease inhibitor by overnight incubation in an aqueous solution) failed to prevent killing of bacteria by apo-hLf (Fig. 1B). Since these inhibitors irreversibly inhibit proteases, these findings indicate that the protease activity required for killing of pneumococci by apo-hLf was probably provided by the pneumococci themselves.

We also observed that the killing of pneumococci by a synthetic LLP (LFN1) (31) could not be inhibited by PMSF. This LLP at 0.3 μM killed log_{10} 0.7 ± 0.2 CFU in 1 h whether or not 7.5 mM PMSF was present (data not shown). This observation that killing by apo-hLf but not by LLP was dependent on serine protease activity suggested that killing by apo-hLf is dependent upon protease activity to release bactericidal peptide(s) from apo-hLf.

Live *S. pneumoniae* strain D39 degraded apo-hLf more efficiently than it degraded holo-hLf. Pneumococci are known to possess several surface-associated proteases and glycosidases. These enzymes have been shown to play an important role in the degradation of host proteins (12, 35, 40, 41). To see if the addition of apo-hLf to the growth medium of growing pneumococci would result in a degradation of apo-hLf, we incubated 2 × 10^6 CFU of actively growing bacteria in 5 ml of THY containing either 0.6 μM apo-hLf or holo-hLf. An aliquot of 500 μl was removed from each incubation mixture when the culture reached the optical density of 0.7 at A_600. The supernatant and cell pellet were separated by centrifugation, run on SDS-PAGE gels, and stained with Coomassie blue. The incubation of apo-hLf in THY with but not without bacteria resulted in the molecular mass of apo-hLf being reduced by about ~12 kDa (68 kDa versus 80 kDa) (Fig. 2A). Even though the entire protein was not completely degraded, the bulk of the protein showed this decrease in the molecular mass, whereas we could still observe some protein at the native molecular mass. It was also observed that holo-hLf was reduced in mass by only 5 kDa (Fig. 2B). When the digested and undigested apo-hLf were excised from the gel and subjected to mass spectrometry, it was observed that although each band showed some heterogeneity in size, the difference in size of the two bands was about 12 kDa.

The fact that apo-hLf was degraded more by incubation with *S. pneumoniae* than was holo-hLf is consistent with the fact that apo-hLf but not holo-hLf can kill pneumococci (31). These observations support the hypothesis that bacterial serine protease releases LLP from apo-hLf but not from holo-hLf and that these LLP are responsible for apo-hLf-mediated killing of pneumococci.

To help determine if the degradation of apo-hLf was due to serine proteases or metalloproteases, apo-hLf was incubated with bacteria in the presence of PMSF (7.5 mM) or with a metalloprotease inhibitor (50 μM EDTA). Incubation with EDTA did not affect the cleavage of apo-hLf (data not shown). However, incubation of apo-hLf with pneumococci in the presence of 7.5 mM PMSF blocked the formation of the 68-kDa peptide typical of the degradation of apo-hLf by the pneumococci.
species of apo-hLf (Fig. 2C). A 75-kDa degradation product of apo-hLf was still observed in the presence of PMSF (Fig. 2C). This 75-kDa product of the 80-kDa parent apo-hLf was similar in mass to the holo-hLf product produced by incubation of holo-hLf with live bacteria in the presence (data not shown) and absence of PMSF (Fig. 2B). In the control reaction mixture containing live bacteria and apo-hLf in the absence of PMSF, we consistently observed the 68-kDa band (Fig. 2C). In addition, a distinct 75-kDa band was also seen (Fig. 2C), indicating incomplete cleavage to the 68-kDa product. This 75-kDa species also appeared to be faintly present under the same conditions (Fig. 2A).

These results demonstrate that in the degradation of the 80-kDa apo-hLf by live bacteria, at least ~7 kDa of the total 12-kDa molecular mass loss could be attributed to serine protease-dependent degradation of apo-hLf. The 5-kDa loss in molecular mass of holo-hLf when it was incubated with live pneumococci was comparable to the protease-independent degradation of apo-hLf. Thus, the 5-kDa protease-independent reduction in mass of both holo- and apo-hLf are likely to be due to glycosidases rather than proteases.

In the experiments illustrated in Fig. 2A to C, apo-hLf was incubated with pneumococci already grown to late log phase (OD = 0.7). In other studies we examined both D39 and EF3030 and observed that both could degrade apo-hLf when grown to a range of ODs from 0.2 to 0.7 (data not shown).

Lack of an effect of rPspA on cleavage of apo-hLf by pneumococci. As little as 0.2 μM recombinant PspA (rPspA) can block killing by 3.1 μM apo-hLf (31). In addition to possibly neutralizing the toxicity of LLP, it was possible that PspA could also protect against apo-hLf-mediated killing by binding to apo-hLf and protecting it from cleavage by proteases. To test this possibility, we attempted to use rPspA to block the degradation of apo-hLf by pneumococcal proteases. Pneumococcal cultures were grown to an optical density of 0.3, washed once with 50 mM Tris buffer (pH 7.5), and then resuspended to an OD of 0.05 in a final volume of 100 μl reaction buffer. Samples were incubated with 3.1 μM lactoferrin, and incubations were carried out in duplicate with and without 1.16 μM rPspA. Samples were removed at 30, 60, 120, and 180 min and subjected to SDS-PAGE and Western blot analysis. Blots were developed with monoclonal antibodies against human lactoferrin.

Detectable cleavage of apo-hLf was first apparent at 1 h following incubation with bacteria, and by 2 h an even greater reduction in the mass of apo-hLf was detected regardless of whether the degraded apo-hLf was recovered in the bacterial pellet or the supernatant (Fig. 3A or B, respectively). Even though this concentration of rPspA far exceeded the concentration required to prevent killing by apo-hLf (31), it failed to show significant inhibition of cleavage of apo-hLf. This finding suggests that PspA did not inhibit killing by apo-hLf by blocking the cleavage of apo-hLf; instead, it prevented killing by binding to apo-hLf and/or to the released LLP and prevented their attack on pneumococcal membranes.

Insertion duplication mutagenesis of prtA resulted in loss of protease activity of S. pneumoniae strains EF3030 and D39. A search of the pneumococcal genome database showed the presence of two serine protease genes, prtA and htrA (48). The proteins encoded by these genes have already been characterized. The gene product of prtA has been shown to be a surface-

![FIG. 2. Cleavage of apo-hLf during incubation with S. pneumoniae D39. Fifty micrograms per milliliter (0.62 μM) apo-hLf and holo-hLf were separately incubated with 10^6 CFU of live D39 pneumococci grown to a density of 0.7 for 2 h under various conditions. The samples were then analyzed by electrophoresis on 4 to 12% SDS-PAGE gradient gels and developed using monoclonal antibody to human lactoferrin. (A) D39 bacteria were washed and incubated with apo-hLf for 2 h prior to separation into the bacterial pellet and supernatant, followed by electrophoresis and Western blotting using monoclonal anti-human lactoferrin. As a control, apo-hLf was incubated for 2 h in THY broth. The difference in mass of the treated versus untreated apo-hLf indicated partial degradation of apo-hLf. (B) holo-hLf incubated with D39 under conditions identical to those for panel A resulted in only a minimal reduction in mass of holo-hLf. (C) Incubation of apo-hLf with D39 in the presence or absence of 7.5 mM protease inhibitor PMSF in 100 mM Tris at pH 7.5 for 1 h at 37°C.](http://iai.asm.org/)

![FIG. 3. Cleavage of apo-hLf by live pneumococci was time dependent and was not inhibited by the presence of rPspA. Fifty micrograms per milliliter (0.62 μM) of apo-hLf was incubated with 100 μl of D39 for 180 min in the presence or absence of 1.16 μM rPspA, and aliquots were removed, separated into supernatant and pellet fractions, and subjected to electrophoresis on 10% Bis-Tris gels followed by Western blotting with antibody to apo-hLf. Cleavage of both free apo-hLf in the supernatant (A) and cell-surface-associated apo-hLf (B) was monitored. After 30 min, the mass of the apo-hLf was still near its native mass of 80 kDa. By 180 min, the mass of the molecule had decreased to about 68 kDa for both the cell surface and cell-bound apo-hLf. By 180 min, a doublet had formed for both cell-bound and cell surface apo-hLf, with the difference in mass of the two forms being about 2 kDa. The presence of 1.16 μM rPspA had no detectable effect on the degree or rate of degradation of apo-hLf.](http://iai.asm.org/)
associated serine protease with a molecular mass of 214 kDa, whereas the gene product of htrA is thought to be a cytoplasmic protein (11). HtrA has been shown to be a heat shock chaperone and is also involved in the competence pathway (40). The role of PrtA, however, is still unknown (11).

The cellular localization of PrtA and the fact that the protease activity of pneumococci was inhibited by a serine protease inhibitor led us to hypothesize that PrtA could be the potential protease responsible for apo-hLf degradation. To see if PrtA may be responsible for the observed cleavage of apo-hLf and thus for activating apo-hLf to kill pneumococci, we prepared mutants lacking PrtA by using insertion duplication to knock out \( \text{prtA} \) in D39 (MJ04.2) and EF3030 (MJ04.1) (Fig. 4). Insertion-duplication mutagenesis was confirmed by using PCR. The mutation should affect only \( \text{prtA} \), since the downstream genes for the phosphotransferase system (PTS) proteins IIA to -C are preceded by independent promoter sequence start sites (48). Thus, neither their expression nor the expression of htrA should have been affected by the disruption of the \( \text{prtA} \) sequence (28, 48).

To see if the mutations in \( \text{prtA} \) blocked the protease activity of the bacteria, the mutant strains and their wild-type parents were incubated with apo-hLf for 1 h and the reaction mixture was run on an SDS-PAGE gel. Consistent with our previous findings, we observed cleavage of apo-hLf by the D39 and EF3030 parental strains (Fig. 5). However, on incubation of apo-hLf with the \( \text{prtA} \)-deficient isogenic mutant strains, no reduction in the size of apo-hLf was observed, indicating the loss of protease activity.

To be sure that the conclusions in Fig. 5A and B were not dependent on a particular bacterial growth phase, we harvested strain D39 and its \( \text{prtA} \) mutant over a range of optical densities (0.2 to 0.6 at \( A_{600} \)) and repeated the original study shown in Fig. 5A. At each optical density, essentially identical results were obtained (data not shown), as was observed with Fig. 5A. These studies made it clear that mutations in \( \text{prtA} \) greatly delayed the degradation of apo-hLf.

**Bactericidal activity of apo-hLf against wild-type pneumococci and their isogenic \( \text{prtA} \) mutants.** To test the possibility that PrtA may play a role in the killing of pneumococci by apo-hLf, the D39 and EF3030 parents and their isogenic \( \text{prtA} \) mutants were incubated with 3.1 \( \mu \text{g}/\text{ml} \) apo-hLf in assay buffer. Although D39 was more sensitive to killing by apo-hLf than EF3030, the presence of the \( \text{prtA} \) mutation made both mutants harder to kill than their respective parental strains (Fig. 6A and B). Interestingly, \( \text{prtA} \) mutant EF3030 was completely resistant to killing by apo-hLf (\( P < 0.001 \)) (Fig. 6B). In the case of EF3030, the size of apo-hLf was observed for incubation with a \( \text{prtA} \)-deficient mutant strain, indicating the loss of protease activity.
of \(\text{prtA}\) mutant D39, however, we observed a significant requirement for PrtA at low concentrations of apo-hLf, but PrtA was not needed for killing at high concentrations of apo-hLf (Fig. 6A). Presumably when the amount of apo-hLf was high enough, other proteases could release a sufficient concentration of toxic fragments to kill the pneumococci.

The effects of the wild type and the \(\text{prtA}\) mutant on degradation of apo-hLf (Fig. 5A) made it clear that given enough time, enzymes other than PrtA could also degrade apo-hLf. The results of the effects of the \(\text{prtA}\) mutation on sensitivity to killing by apolactoferrin made it clear that among the enzymes that can degrade apo-hLf, PrtA plays a critical role in activating apo-hLf and either produces the bactericidal fragments or makes a cleavage that allows other enzymes to create the fragments. However, in the case of D39 at least one other protease (48) is probably also able to activate apo-hLf. These results and our findings that serine protease inhibitors completely inhibited killing of D39 by a \(3.1 \times 10^{-3}\) g/ml concentration of apo-hLf (Fig. 1) are evidence that serine proteases and PrtA in particular can play a critical role in activating apo-hLf.

**Bactericidal activity of apo-hLf against a \(\text{pspA} \text{prtA}\) double mutant.** It has been previously reported that PspA’s ability to protect pneumococci from killing by apo-hLf is directly correlated with PspA’s ability to bind apo-hLf. Experiments performed in this study have demonstrated a role for PrtA in the killing of wild-type pneumococci by apo-hLf. Since PspA- pneumococci are much more sensitive to killing by apo-hLf, we wanted to see if the \(\text{prtA}\) mutant would also have an effect on killing of these highly susceptible bacteria. Our data indicate that the \(\text{prtA pspA}\) double mutant of EF3030 (MJ04.11) was killed much less efficiently than the \(\text{pspA}\) mutant of EF3030. In fact, the double mutant showed a level of killing not distinguishable from that of the \(\text{prtA}\) mutant itself. (Fig. 7). These findings supported our conclusion that PrtA expression is important for killing of pneumococci by apo-hLf.

**Recombinant PrtA cleaved human lactoferrin.** Recombinant PrtA was expressed as a 113-kDa, glutathione S-transferase (GST) S-tagged and His-tagged protein (Fig. 8A). The protease activity of rPrtA was confirmed by a casein zymogram, with 5 U of trypsin as a positive control. Protease activity at the expected molecular mass of PrtA (113 kDa) was demonstrated by the presence of characteristic clear zones on Coomassie blue-stained gels (data not shown). Following overnight incubation of 10 \(\mu\)g/ml apo-hLf with an equal concentration of PrtA, we observed that PrtA can partially degrade apo-hLf (Fig. 8B). The cleavage of apo-hLf by PrtA was time dependent, since longer incubations resulted in a smaller residual mass of the apo-hLf. After a 180-min incubation, the Western

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**FIG. 6.** Role of PrtA in killing by apo-hLf. Disruption of the \(\text{prtA}\) gene in \(S.\ pneumoniae\) capsule type 2 strain D39 (A) or type 19F strain EF3030 (B) resulted in the inhibition of bactericidal activity of apo-hLf, demonstrating the role of protease in killing by apo-hLf. A reduction in killing of the \(\text{prtA}\)-negative mutant compared to that of the wild-type strain further confirmed our findings, presented in Fig. 5, demonstrating the inhibition of cleavage of lactoferrin in the absence of \(\text{prtA}\).

**FIG. 7.** Bactericidal activity of apo-hLf on \(\text{pspA}\)-negative strains and \(\text{prtA pspA}\) double-negative strains of \(S.\ pneumoniae\) EF3030. Even in the absence of PspA, a lack of \(\text{prtA}\) still blocked killing. Log CFU obtained from killing of \(\text{prtA pspA}\) double-negative mutants were not significantly greater than those from the killing of the \(\text{prtA}\)-deficient mutant alone. The \(\text{pspA}\)-negative strain showed significant killing compared with the wild-type strain and either or the \(\text{prtA}\)-negative mutants (\(P < 0.002\)).

**FIG. 8.** Recombinant PrtA degrades apo-hLf. (A) Coomassie blue-stained gel showing purified recombinant PrtA (113 kDa, as expected) isolated by Talon metal affinity resin. (B) Coomassie blue-stained gel of 10 \(\mu\)g/ml of apo-hLf incubated with 10 \(\mu\)g/ml of rPrtA overnight. (C) Western blot using polyclonal antilactoferrin antibody demonstrated cleavage of apo-hLf by recombinant PrtA over a period of 3 h. A total of 10 \(\mu\)g/ml of apo-hLf was incubated with or without 10 \(\mu\)g/ml of PrtA in a 50 mM Tris-HCl (pH 7.5) and 50 mM CaCl\(_2\) buffer at 37°C for 2 h.
increased the bactericidal activity of apo-hLf when it was added to EF3030 (not shown). (B) Incubation of apo-hLf with rPrtA for 0.6, 1.0, and 16 h prior to incubation with EF3030. Prior incubation of apo-hLf with rPrtA increased the bactericidal activity of apo-hLf when it was added to EF3030 (P = 0.001).

\[\text{FIG. 9. rPrtA enhances the bactericidal activity of apo-hLf. (A) Killing of the isogenic prtA-deficient mutant MJ04.1 of EF3030 and the EF3030 parent with apo-hLf in the presence and absence of recombinant PrtA (P = 0.001). The killing of the EF3030 parent with 100 \mu g/ml (1.25 \mu M) apo-hLf in the absence of added rPrtA is shown for comparison. The addition of 50 \mu g rPrtA to the PrtA mutant strain completely restored the strain’s ability to be killed by apo-hLf, to the level of that seen with the EF3030 parent. Controls were incubated with the same concentration of PrtA in the absence of apo-hLf. No killing resulted from the incubation of the PrtA mutant bacteria with rPrpA in the absence of apo-hLf (data not shown). (B) Incubation of apo-hLf with rPrtA for 0.6, 1.0, and 16 h prior to incubation with EF3030. Prior incubation of apo-hLf with rPrtA increased the bactericidal activity of apo-hLf when it was added to EF3030 (P = 0.001).}\]

Killing of EF3030 and PrtA mutant EF3030 (MJ04.1) by apo-hLf in the presence of recombinant PrtA. In the studies shown in Fig. 6 and 7, we observed that pneumococcal strains deficient in PrtA expression were relatively resistant to killing by apo-hLf compared to wild-type strains. To confirm that the inability of strains with this mutation to be killed was because of the loss of PrtA expression, we performed a complementation experiment to see if adding back rPrtA would allow a mutant strain to be efficiently killed by apo-hLf. The pneumococcal strains EF3030 and MJ04.1 were treated with 3.1 \mu M apo-hLf in the presence or absence of 50 \mu g/ml of rPrtA. As expected, although the PrtA mutant strain MJ04.1 could not be efficiently killed by apo-hLf alone, its ability to be killed was completely restored by adding 50 \mu g/ml of recombinant PrtA (P < 0.001) (Fig. 9A). When MJ04.1 was treated with rPrtA in the absence of apo-hLf, no killing was observed (data not shown).

Ability of incubation with rPrtA to increase the bactericidal capacity of a solution containing apo-hLf. LLP are known to be more bactericidal on a molar basis than apo-hLf (31). Thus, if the killing of pneumococci by apo-hLf is mediated by a fragment of apo-hLf (LLP) that is cleaved from apo-hLf by PrtA, then the prior incubation of apo-hLf with rPrtA should increase the bactericidal capacity of apo-hLf. For this study we treated EF3030 with a subbactericidal concentration of apo-hLf or with the same concentration of apo-hLf after it had been digested for various time points with rPrtA. A final concentration of 1.25 \mu M apo-hLf was incubated with 50 \mu g/ml of rPrtA for 0.6, 1, and 16 h. Bacteria were then treated with a 1.25 \mu M concentration of this treated apo-hLf. As a control, EF3030 was also treated with a total of 1.25 \mu M native apo-hLf in the absence of rPrtA. It was observed that the apo-hLf that was incubated with rPrtA for the longest period of time (16 h) was the most bactericidal, resulting in 0.65 logs more killing than apo-hLf alone (P = 0.001), a finding consistent with the release of active LLP from apo-hLf. As in Fig. 9A, the incubation of EF3030 with PrtA in the absence of apo-hLf did not result in detectable killing (data not shown).

Identification of the cleavage site of PrtA in lactoferrin. We attempted to identify the site in lactoferrin that is cleaved by PrtA. All of the bactericidal peptides of lactoferrin are known to be derived from the N-terminal end of the protein and are contained in the N-terminal 47 amino acids (36, 51). It was therefore hypothesized that cleavages of lactoferrin that would include all or most of the first 47 amino acid were responsible for yielding the peptide(s) of apo-hLf that is bactericidal to S. pneumoniae. A total of 10 \mu g/ml of apo-hLf was incubated with or without 10 \mu g/ml of PrtA in a 50 mM Tris-HCl (pH 7.5) and 50 mM CaCl₂ buffer at 37°C for 2 h. Untreated apo-hLf and PrtA-treated apo-hLf were trypsinized and subjected to LC/MS. Results of LC/MS identified a cleavage site between Val₁⁷ and Ala₃₉ of the tryptic peptide LRVPVAAEVYGT, thus generating the peptide AAEVYGT. To confirm this cleavage, trypsin-generated peptides of apo-hLf and PrtA-digested apo-hLf were analyzed by LC–multiple-reaction ion monitoring (MRM)–MS. Individual channels were set up to monitor the parent tryptic peptide, LRVPVAAEVYG, and the PrtA-cleaved peptide, AAEVYG. The molecular ions for each peptide were selected in the first quadrupole and fragmented in the second quadrupole. Specific fragment ions unique to each peptide were selected in the third quadrupole (Table 3). Results of MRM analysis indicated that PrtA cleaved a 78-amino-acid fragment from the N terminus of apo-hLf (Fig. 10B), whereas no such product was identified in the absence of digestion with PrtA (Fig. 10A). This was the only major cleavage site found in the protein under these conditions. Scanning of the C-terminal region of lactoferrin did not show any evidence of additional cleavage. Even though the cleavage did not occur in the expected first 47 amino acids, which could have yielded the traditional lactoferricin peptide, in the N-terminal region, the fragment obtained by cleavage...
between residues 78 and 79 includes the first 47 amino acids of apo-hLf, where the bactericidal activity of lactoferrin has been shown to reside (19). It is possible that once this fragment is released, it undergoes a heterogeneous pattern of cleavage, which produces many different bactericidal fragments but no major cleavage sites that we were able to identify.

### DISCUSSION

These studies have addressed the hypothesis that the killing of pneumococci by apo-hLf is dependent on the cleavage of an N-terminal LLP (peptide) from apo-hLf by pneumococcal proteases, primarily PrtA, and that it is this cleaved LLP (or a smaller LLP derived from it) that actually kills the pneumococci. This hypothesis is supported by our observations, as follows: (i) serine protease inhibitors block killing by apo-hLf but not by LLP, (ii) incubation of apo-hLf with live pneumococci or the pneumococcal protease PrtA results in protease-dependent removal of ~8 kDa, (iii) pretreatment of apo-hLf with PrtA makes the mixture of apo-hLf and PrtA severalfold more bactericidal, (iv) mutant pneumococci lacking PrtA are poorly able to degrade apo-hLf and are not efficiently killed by apo-hLf, (v) PrtA can complement the prtA mutation and restore susceptibility to apo-hLf, (vi) holo-hLf, which is not bactericidal, does not exhibit a serine protease-dependent loss of 8 kDa in mass, and (vii) a major PrtA cleavage site in apo-hLf between amino acids 78 and 79 removes the N-terminal 78 amino acids (~8 kDa) of apo-hLf, which includes the sequences of the known bactericidal LLP.

It is well established that the LLP from apo-hLf that are produced in the lab by enzymatic attack or weak acid hydrolysis can be much more bactericidal than apo-hLf itself (31, 39, 49, 54). Using two synthesized LLP, we have confirmed that LLP can be more bactericidal to pneumococci than apo-hLf (22). It has been proposed that these antibacterial peptides act at or through the bacterial membrane (1, 4, 16, 27). It seems likely that these short peptides should be able to transit the cell wall and reach the bacterial membrane much more efficiently than intact apo-hLf. These considerations led us to originally hypothesize that killing by apo-hLf is dependent on its cleavage and the consequent release of anti-bacterial LLP.

Holo-hLf has been shown to generally lack bactericidal activity against pathogens and to be less readily cleaved by protease than is apo-hLf (9), and our present findings indicate that apo-hLf is more readily cleaved by incubation with pneumococci than is holo-hLf. It is thought that apo-hLf is more structurally flexible than holo-hLf, allowing its N1 lobe to exist in an open conformation, which in turn may contribute to the greater susceptibility of apo-hLf than holo-hLf to proteolytic cleavage (2, 8, 9).

In earlier work, we demonstrated that PspA, which binds apo-hLf, could block killing by apo-hLf and two LLP (31). In this study we observed that rPspA failed to block cleavage of apo-hLf by pneumococci, a result suggesting that PspA’s ability to prevent killing by apo-hLf resides primarily in its ability to bind and thus block killing by the LLP cleaved from apo-hLf by PrtA and possibly other pneumococcal proteases. This conclusion is consistent with our observations that apo-hLf cannot kill if it is not first cleaved by a serine protease and that killing by a synthetic LLP is not inhibited by PrtA.

Proteases in bacteria are known to perform several physiological and pathogenic functions, including the acquisition of nutrients, the turnover of proteins, enzyme modification and

### TABLE 3. Multiple reaction monitoring of lactoferrin peptide

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>m/z</th>
<th>Parent ions</th>
<th>Daughters</th>
<th>Daughter ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEVYGTER</td>
<td>498.2 (2+)</td>
<td>853.4</td>
<td>y7</td>
<td>724.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>625.3</td>
<td>y5</td>
<td></td>
</tr>
<tr>
<td>LRPVAAEVYGET</td>
<td>730.9 (2+)</td>
<td>1191.6</td>
<td>y11</td>
<td>737.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>999.6</td>
<td>b5</td>
<td></td>
</tr>
</tbody>
</table>

*Ionic charge is given in parentheses.*
processing, the destruction of proteins involved in host immu-
nity, and the activation of host proteases (45). The iron-binding proteins of the host, such as lactoferrin and transferrin, are major constituents of upper airway secretions and also play a significant role in modulation of the host immune response (21). Proteases expressed by some species of bacteria enable the bacteria to use holo-lactoferrin or transferrin as a source of iron (18, 20). Streptococcus pneumoniae, however, is not able to acquire iron from holo-lactoferrin or transferrin and instead acquires iron from hemin (46), a finding that we have confirmed using pneumococci grown under conditions of iron stress (S. Mirza and D. E. Briles, unpublished data).

In this study, we demonstrated a role for the pneumococcal surface-associated serine protease PrtA in killing by apo-lactoferrin and have provided evidence that PrtA is able to cleave at least one host protein, apo-lactoferrin. It is possible that PrtA may have many important roles that would provide a selective advantage to the pneumococci that outweigh the fact that PrtA cleaves apo-lactoferrin at the bacterial surface to release LLP, which can cause bacterial death. That apo-lactoferrin can be cleaved to yield LLP, using a broad range of different proteases (31, 39, 49, 54), may partially explain why bacteria have failed to evolve proteases that serve the functions that the bacteria need without also releasing LLP.

Incubation of apo-lactoferrin with pneumococci reduced its mass by ~12 kDa. In the presence of protease inhibitors, this reduction was only about ~5 kDa, indicating that the protease-dependent reduction in mass was about ~7 kDa. When holo-lactoferrin, which is not bactericidal, is incubated with pneumococci, its reduction in mass was also ~5 kDa, and this reduction in mass was not affected by the protease inhibitor. When PrtA was incubated with apo-lactoferrin, the reduction in mass of the apo-lactoferrin on SDS gels was ~8 kDa (not measurably different from the 7 kDa on our gels). This 8-kDa reduction in mass was consistent with the cleavage we detected by MRM and QTRAP mass spectrometry between the amino acids Val78 and Ala79. These results suggest that the serine protease-dependent degradation of apo-lactoferrin removes ~8 kDa. Even though the conventional bactericidal peptides have been identified in the first 47 amino acids of the N-terminal region of apo-lactoferrin, the possibility that peptides from other regions could potentially exert bactericidal effects is also plausible (36, 51).

The protease-independent reduction of ~5 kDa in mass is probably due to glycosidases or nonserine proteases and does not involve the N-terminal 8 kDa of lactoferrin. Both apo- and holo-lactoferrin have oligosaccharides at positions 137 and 478, and the 5-kDa loss in mass is not protease dependent and is not expected to be dependent on whether lactoferrin contains or lacks iron. It is possible, but far from certain, that cleavage of these polysaccharides explains part or all of the serine protease-independent 5-kDa loss in mass caused by incubating lactoferrin with live pneumococci.

We have failed after many attempts with many different methods to detect a free peptide of ~8 kDa resulting from incubation of apo-lactoferrin with PrtA or with live pneumococci, and we suspect that this may be because the original 8-kDa fragment following incubation of apo-lactoferrin may have been further degraded, yielding a diverse group of degradation products. On the internal disulfides between cysteines 9 and 45 and between cysteines 19 and 36 (42), any single cleavage in the N-terminal region between positions 45 and 9 would not break the 78-amino-acid (~8-kDa) N-terminal fragment into parts. It is thus likely that several subsequent cleavages of the 78-amino-acid fragment may have occurred. In the studies shown in Fig. 5, we showed that incubation of apo-holo-lactoferrin with pneumo-
cocci for no more than 10 to 30 min was required for its cleavage by PrtA to be visualized. With significantly longer incubation times, it became apparent that non-PrtA enzymes could also degrade apo-lactoferrin. It is possible that non-PrtA proteases may also be responsible for cleavages in the 8-kDa fragment once it is released from apo-lactoferrin by PrtA.

Although the substrate specificity of PrtA was not tested, Val78 at position P1 is characteristic of thermolysins, which preferentially cleave sites with either Ala or bulky or aromatic residues (Ile, Leu, Val, Met, and Phe) in position P1 (29). This single cleavage could account for the about 8-kDa mass shift seen in Fig. 8, where rPrtA was incubated with lactoferrin. The fact that a less discrete and progressive mass shift in apo-lactoferrin was observed during incubation with live pneumococci is probably the result of degradation by polysaccharide-cleaving en-
ymases and other proteases that may cleave both prior to and subsequent to PrtA.

Apolactoferrin may be a bit of a “Trojan horse.” The bac-
teria readily process it with enzymes used for their virulence and nutrition, only to create instead a fragment that is bacte-
cidal. Our results with pneumococci suggest that microbial proteases may be required for an attack by apo-lactoferrin on other microbes. Having the generation of LLP be dependent on an enzyme of the microbe being attacked may help ensure that the amphipathic LLP are produced very near the microbial sur-
face, where they would have the least chance of being lost through interactions with other molecules in the host milieu.

PrtA has been reported to be a surface-exposed virulence factor important in invasive pneumococcal disease and could thus become a vaccine candidate (11). Our present findings, however, suggest that it may be unwise to use PrtA in a vaccine, since antibodies to it could inhibit killing of S. pneumoniae by apo-lactoferrin.

ACKNOWLEDGMENTS

We are grateful to G. Zysk for providing pJDC9 containing a cloned prtA gene fragment, which was used to make some of our mutants, and to Janice King for frequent advice and encouragement.

These studies were supported by NIH grants AI21548 and AI053749 and The National Research Council of Korea, WCU R33-10045. The mass spectrometer used in this study was purchased with funds from a NCRR Shared Instrumentation Grant (S10 RR19231-01, S.B., principal investigator).

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Editor: J. N. Weiser