A novel streptococcal surface protease promotes virulence, resistance to opsonophagocytosis, and cleavage of human fibrinogen

Theresa O. Harris,1 Daniel W. Shelver,1 John F. Bohnsack,2 and Craig E. Rubens1

1Division of Infectious Disease, Children’s Hospital and Regional Medical Center, and University of Washington, Seattle, Washington, USA
2Department of Pediatrics and Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah, USA

Group B streptococcus (GBS) is an important human pathogen. In this study, we sought to identify mechanisms that may protect GBS from host defenses in addition to its capsular polysaccharide. A gene encoding a cell-surface–associated protein (cspA) was characterized from a highly virulent type III GBS isolate, COH1. Its sequence indicated that it is a subtilisin-like extracellular serine protease homologous to streptococcal C5a peptidases and caseinases of lactic acid bacteria. The wild-type strain cleaved the α chain of human fibrinogen, whereas a cspa mutant, TOH121, was unable to cleave fibrinogen. We observed aggregated material when COH1 was incubated with fibrinogen but not when the mutant strain was treated similarly. This suggested that the product(s) of fibrinogen cleavage have strong adhesive properties and may be similar to fibrin. The cspa gene was present among representative clinical isolates from all nine capsular serotypes, as revealed by Southern blotting. A cspa mutant was ten times less virulent in a neonatal rat sepsis model of GBS infections, as measured by LD50 analysis. In addition, the cspa mutant was significantly more sensitive than the wild-type strain to opsonophagocytic killing by human neutrophils in vitro. Taken together, the results suggest that cleavage of fibrinogen by CspA may increase the lethality of GBS infection, potentially by protecting the bacterium from opsonophagocytic killing.


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Address correspondence to: Craig E. Rubens, 4800 Sand Point Way NE, Seattle, Washington 98105, USA.
Phone: (206) 526-2073; Fax: (206) 527-3890; E-mail: cruben@chmc.org.
Theresa O. Harris and Daniel Shelver contributed equally to this work.

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Nonstandard abbreviations used: group B streptococcus (GBS); cell-surface–associated protein (CspA); polymorphonuclear leukocytes (PMNs); group A streptococcus (GAS); Todd-Hewitt broth (THB); matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); cell envelope–associated protease (CEP).
cytosis. CspA shows homology to a family of proteases that include C5a proteases of pathogenic streptococci (11) as well as caseinases expressed by nonpathogenic Gram-positive cocci (12). Surprisingly, we observed that CspA does not have enzymatic activity against C5a in vitro and the presence of the cspA gene was not required for casein degradation. However, the cspA gene was required for GBS cleavage of human fibrinogen, which indicates that CspA is active as a protease. Mutants that failed to express cspa displayed a significantly decreased GBS virulence in a neonatal rat model of infection and displayed increased sensitivity to opsonophagocytosis. Our findings provide evidence that CspA is a novel, surface-localized protease that plays an important role in GBS pathogenesis as an antiphagocytic surface factor.

Methods

Bacterial strains. COH1 is a highly encapsulated type III GBS strain, originally isolated from the blood of a septic newborn (13). Other GBS clinical strains used in this work included type Ia strains B523 (14), A909 (14), and Chan55; type Ib strains DK14, DK15, and 80-481; type II strains 78-471 (15) and DK23 (14); type III strains COH31 (9), D136C (14), and M781 (14); type IV strain CNCTC1/82 (14); type V strains B201 and CNCTC 10/84 (14); type VI strain NT6 (14); type VII strain 87-603; and type VIII strain JM9 (kindly provided by Pat Ferrieri, University of Minnesota, Minneapolis, Minnesota, USA). COH1-13 is an acapsular Tn916 Δcspa mutant of COH1 (16).

Media, chemicals, and culture of bacterial strains. Escherichia coli and GBS were grown in Luria broth and Todd-Hewitt broth (THB), respectively. Concentrations of antibiotics for selection included ampicillin (Amp, 75 µg/ml), erythromycin (Erm, 400 µg/ml for E. coli and 10 µg/ml for GBS), or chloramphenicol (Cam, 10 µg/ml). For the culture of GBS in human plasma, plasma was obtained from healthy human donors who provided consent. GBS was grown to OD600 of 0.6 in THB, washed twice in an equal volume of PBS, and resuspended in plasma at a concentration of approximately 1000 CFU/ml. Growth was then monitored over a 6-hour period, duplicate dilutions were plated, and doubling time was calculated.

DNA and RNA methods. Standard procedures for cloning, sequencing, Southern blotting, Northern blotting, and PCR amplification were used (17). RNA was isolated by the method of Yim and Rubens (18). Antisense digoxigenin-labeled probes were used for Northern blot procedures as recommended by the manufacturer (Roche Molecular Biochemicals, Indianapolis, Indiana, USA).

Identification and cloning of the cspa locus. A portion of the cspa open reading frame was originally isolated by analysis of the transposon insertion site from a Tn916AE mutant of strain COH1 and was used as a probe to clone the entire cspa gene. Overlapping ClaI and XbaI restriction fragments (Figure 1) that bear cspa were identified by Southern analysis of COH1 genomic DNA and cloned into pBSKS- (Stratagene, La Jolla, California, USA) using standard techniques. E. coli DH5α clones harboring the desired GBS inserts were identified by colony blots using the probe mentioned above. Clones containing either a ClaI fragment (TOH37 containing plasmid pTH2) or an XbaI fragment (TOH50 containing plasmid pTH5) were further analyzed. The cloned cspa gene present on plasmid pTH5 contains a spontaneous mutation, in comparison with the chromosomal cspa sequence of the wild-type isogenic strain, COH1; this mutation is predicted to terminate translation prematurely at Leu-1121. The sequences described in this work have been deposited in GenBank under accession number AY162834.

Construction of a cspa::erm mutation in GBS. To perform allelic replacement mutagenesis of cspa, we subcloned cspa to pVE6007, a broad-host-range plasmid that replicates at 28°C but not at 37°C (19). A 5.4-kb PCR product of cspa was amplified from COH1 genomic DNA, digested with BamHI and XbaI, and cloned into BamHI/XbaI-digested pVE6007, generating intermediate plasmid pTH19. Approximately 3.6 kb of cspa sequence (corresponding to CspA peptide residues 323–1536) flanked by HindIII sites was subsequently replaced with the erm element from pCER1000 (20). The final construct, pTH21, was transformed into COH1 as described (21). A strain that bears a replacement of cspa with the erm element was obtained by curing the plasmid, as described by Yim and Rubens (22), and was designated TOH121. The presence of the desired mutation on the chromosome of TOH121 was verified by Southern blotting.

Phenotypic and LD50 virulence assays. Analysis of cell- associated type III GBS capsule (23), beta-hemolysin, CAMP factor (a factor that destabilizes and lyses erythrocyte membranes) (24), hyaluronidase (25), and hippuricase expression was performed as described (26). Caseinase activity was evaluated by several means. First, caseinase activity was measured by growing GBS overnight on THB+5% milk agar plates. Second, caseinase activity was measured by incubating GBS culture supernatants, mutanolysin extracts, or whole cells with purified, caseinate assay-grade casein (Sigma-Aldrich, St. Louis, Missouri, USA). Adherence and invasion assays of A549 cell monolayers (type II lung epithelial cells) were performed as described (27). C5a protease activity of individual strains was determined by the ability of GBS to inhibit C5a-stimulated adherence of human PMNs to gelatin-coated tissue-culture wells as described (28). The virulence of isogenic cspa−/− strains was compared using a neonatal rat model of lethal GBS infection by LD50 analysis as described previously (29); statistical analysis for the LD50 data was performed with the Wilcoxon matched-pair signed-ranks test. All animals were maintained according to institutional, state, and federal guidelines.

Recombinant Cspa–GST fusion protein production and generation of Cspa antibody. A Cspa–GST fusion protein was constructed for use as an immunogen. A C-terminal portion of Cspa that lacks the putative catalytic domain was
amplified from COH1 chromosomal DNA with primers TCGGATCCGCTACTGCTCTAGTT and TTAAGTCGACGTAATGATGCCTTGCTCTA, which incorporate BamHI and SalI sites for cloning. Plasmid pGEX-4T-3 (Amersham-Pharmacia Biosciences, Piscataway, New Jersey, USA) was digested with BamHI and SalI and ligated to the PCR product, which was also digested with BamHI and SalI. The CspA-GST fusion protein formed inclusion bodies; after solubilization and SDS-PAGE, CspA was excised from polyacrylamide gels and fragmented as described (30). This preparation was used to immunize a New Zealand white rabbit previously shown to lack antibody to the fusion protein.

Western blot analysis of CspA. CspA was released from the periplasmic space of TOH50 (E. coli DH5α harboring plasmid pTH5) by an osmotic shock procedure (31). GBS cell-surface–associated proteins were extracted by treatment with mutanolysin and subjected to SDS-PAGE (32). Proteins were transferred to Immobilon-P (Millipore Inc., Bedford, Massachusetts, USA); primary antibody and secondary horseradish peroxidase–conjugated antibody were used at dilutions of 1:500 and 1:1000, respectively. The SuperSignal reagent (Pierce Biotechnology Inc., Rockford, Illinois, USA) was utilized for the chemiluminescent detection of Western blots. Fibrinogen degradation assays. Purified human fibrinogen, depleted of fibronectin and plasminogen, was purchased from Enzyme Research Laboratories (South Bend, Indiana, USA). To assay fibrinogen degradation, GBS strains COH1 and TOH121 were grown to stationary phase, washed once in PBS, concentrated 20-fold, and resuspended in PBS. Fibrinogen was then added at a concentration of 0.63 mg/ml. The fibrinogen/cell suspension was incubated with slow rotation at 37°C. After overnight incubation, the trypsin solution was added to the dehydrated gel fragments, incubated for 45 minutes at 4°C, and then incubated overnight at 37°C. After overnight incubation, the trypsin solution was removed, and the gel slices were extracted twice with 200 µl of 5% formic acid and 50% acetonitrile. The trypsin solution was pooled with the extraction solution and evaporated under vacuum. Ten microfilters of 5% acetonitrile and 0.5% acetic acid was added, and 0.6 µl was spotted on the target. After this, mass spectra were acquired using a BIFLEX III mass spectrometer (Bruker, Billerica, Massachusetts, USA).

Opsonophagocytosis assays. Opsonophagocytosis assays were performed using serum and neutrophils that were obtained with consent from nonimmune humans, as previously described (33). All assays were performed in triplicate, and controls included samples with heat-inactivated sera (56°C for 30 minutes) and without PMNs.

Results
It is likely that many factors contribute to the virulence of GBS (29). In order to identify novel virulence factors, we performed a screen to identify transposon mutants with reduced virulence in GBS. In the process of screening Tn916 ΔE mutants of the highly virulent type III GBS isolate, COH1, we identified a gene with homology to cell-surface–associated proteases. The open reading frame, which we designated cspA (Figure 1), encodes a 1,571–amino acid protein and displays homology to several extracellular serine proteases from the subtilase family. The greatest similarity of CspA (51.2% identity and 58.3% similarity) was to PrtS, which is an extracellular caseinase produced by S. thermophilus (12). The next highest similarity was to a putative extracellular protease (39% identity and 55% similarity) from the GAS genome sequence (34). The third highest similarity (45% similarity and 36% identity) was to ScpA (35) and ScpB.
Northern blot analysis of \textit{cspA} expression in COH1 and TOH121. Standard Northern blots were performed using equivalent amounts of RNA (5 µg) from the indicated strains. (a) Blot developed using \textit{cspA} probe. Lane 1, COH1 at OD\textsubscript{600} = 0.3 in THB; lane 2, COH1 at OD = 0.6; lane 3, COH1 at OD = 1.7; lane 4, TOH121 at OD = 0.3; lane 5, TOH121 at OD = 1.7. (b) Blot developed using \textit{sbrA} probe. Lane 1, COH1 at OD = 0.6; lane 2, TOH121 at OD = 0.6. Migration of RNA size standards is indicated (in kb) on the right.

\textbf{Figure 2}

Northern blot analysis of \textit{cspA} expression in COH1 and TOH121. Standard Northern blots were performed using equivalent amounts of RNA (5 µg) from the indicated strains. (a) Blot developed using \textit{cspA} probe. Lane 1, COH1 at OD\textsubscript{600} = 0.3 in THB; lane 2, COH1 at OD = 0.6; lane 3, COH1 at OD = 1.7; lane 4, TOH121 at OD = 0.3; lane 5, TOH121 at OD = 1.7. (b) Blot developed using \textit{sbrA} probe. Lane 1, COH1 at OD = 0.6; lane 2, TOH121 at OD = 0.6. Migration of RNA size standards is indicated (in kb) on the right.

\textbf{Figure 3}

Western blot analysis of CspA expression in \textit{E. coli} and GBS strains using anti-CspA sera. Lane 1 shows periplasmic extract from \textit{E. coli} DH5\textsuperscript{α} containing pBS (negative control); Lane 2 shows periplasmic extract from \textit{E. coli} DH5\textsuperscript{α} containing pTH5 (XbuI fragment bearing \textit{cspA} in pBSK\textsuperscript{−}; Stratagene). Note that the lower migration of CspA in periplasmic extracts is due to a mutation in pTH5 that prematurely terminates translation (see Methods). Lanes 3–6 show mutants in extracted GBS surface proteins from COH1 (lane 3), TOH121 (\textit{cspA}–, lane 4), TOH97 (\textit{sbrA}–, lane 5), and TOH144 (\textit{cspA}–, \textit{sbrA}–, lane 6). Migration of molecular mass markers (in kDa) is indicated on the left.
Figure 4
Functional assay for C5a protease activity. Shown is the percent adhesion by human PMNs to gelatin-coated tissue-culture wells after the indicated GBS strains were incubated with recombinant human C5a (28). As controls, buffer alone (buffer) or 100 ng/ml of untreated C5a (C5a) were incubated without bacteria before exposure to PMNs. GBS strains that were tested were COH1 (positive control, C5a-ase activity), TOH97 (negative control, CspA, scpB), TOH121 (CspA, scpB), and TOH144 (CspA, scpB).

CspA is a surface-associated, cell-wall–anchored protein. In addition to predicting that CspA functions as a protease, the sequence of CspA suggests that it is secreted and subsequently anchored to the cell surface. A 35-residue signal peptide within the N-terminal end of CspA was identified using SIGSEQ (40) (Figure 1). A classic C-terminal cell-wall attachment site sequence (LPKTG) characteristic of Gram-positive surface-associated proteins (41) was located at amino acids 1536–1540 (Figure 1). To investigate the hypothesis that CspA is a surface-attached protein, we determined the subcellular localization of CspA by Western blot analysis. Antibody was raised to a GST–CspA fusion protein expressed from E. coli (see Methods), and the antibody was used to test different cellular fractions of GBS for the presence of CspA. Periplasmic extracts (see Methods) of E. coli strain TOH50 (bearing plasmid pTH5; see above) reacted strongly with the antibody raised to GST–CspA. Plasmid pTH5 contains a mutation in the CspA coding region (in comparison with the cspA gene of the wild-type isogenic strain, COH1) that prematurely terminates translation at Leu-1121; this accounts for the lower molecular mass observed for the TOH50 extracts in comparison with wild-type CspA. Proteins from culture supernatants of COH1 did not react with the antibody, even when concentrated 10-fold (data not shown). In contrast, Western blots of mutanolysin-extracted surface proteins from both COH1 and TOH97 (CspA, scpB) revealed two protein bands of molecular masses 142 and 80 kDa (Figure 3). The migration of COH1-derived CspA on SDS-PAGE was anomalous, since it corresponded to a lower molecular mass (142 kDa) than predicted by sequence analysis for mature CspA (153 kDa). No cross-reactive bands were seen at the 142-kDa position for the cspA mutants (TOH121 and TOH144), confirming that the antibody does react with wild-type CspA from GBS. The results of the sequence analysis of CspA taken together with the Western blot data indicate the CspA is a surface-localized protein.

Table 1
Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td><strong>S. agalactiae</strong></td>
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<td></td>
</tr>
<tr>
<td>COH1</td>
<td>Type III GBS isolate from neonate with septicemia; highly virulent; wild-type reference strain</td>
<td>13</td>
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<tr>
<td>TOH121</td>
<td>cspa::erm derivative of COH1; Erm’</td>
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<td>scpB::kan derivative of TOH50; Erm’</td>
<td>54</td>
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<tr>
<td>TOH144</td>
<td>cspa::erm derivative of TOH50; Erm’</td>
<td>this work</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td></td>
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<td>General cloning strain</td>
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<td>TOH50</td>
<td>DH5α bearing plasmid pTH5</td>
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<tr>
<td><strong>Plasmid</strong></td>
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<td>pBSKS</td>
<td>General cloning vector</td>
<td>Stratagene</td>
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<td>pTH2</td>
<td>pBSKS bearing Clal DNA fragment of cspa; Amp’</td>
<td>this work</td>
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<td>pTH5</td>
<td>pBSKS bearing XbaI DNA fragment containing cspa; Amp’</td>
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<td>pTH21</td>
<td>Plasmid used for insertion mutagenesis of cspa; derivative of pVE6007; Cm’; Erm’</td>
<td>this work</td>
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</table>
**Figure 5**

Caseinase activity assay. GBS strain COH1 was grown to stationary phase in THB, washed in PBS, concentrated 20-fold, and resuspended in PBS. A saturated solution of protease assay-grade casein (Sigma-Aldrich) was prepared, and 0.2 ml of bacteria was mixed with 0.3 ml of casein. A mock reaction with PBS (no bacteria) was also prepared. The mixtures were incubated for 24 hours at 37°C, and an aliquot was removed for SDS-PAGE. Molecular-weight markers are depicted on the left. Lane 1 shows the PBS control, lane 2, COH1 after 0 hours of incubation, and lane 3, COH1 after 24 hours of incubation. Migration of molecular mass markers (in kDa) is indicated on the left.

CspA does not function as a C5a protease. Given the strong similarity of CspA to ScpB, the C5a peptidase of GBS, we hypothesized that CspA may also be active as a C5a protease. We measured C5a-ase activity (Figure 4) with a functional assay described previously (11). Briefly, GBS was preincubated with recombinant human C5a, purified human PMNs were added, and C5a-stimulated PMN adherence to gelatin-coated plastic was measured. COH1 (wild-type, scpB+) served as a positive control, and TOH97 (scpB−) and another scpB− strain, GW (32), served as negative controls. The effect of the cspA mutation was measured both in the presence and absence of the GBS C5a protease (ScpB) by comparing TOH121 (scpB+, cspA+) and TOH144 (scpB−, cspA+) with the controls. Preincubation of C5a with COH1 or TOH121 abolished C5a activity, and the C5a protease activities of the strains were similar (Figure 4). In contrast, C5a protease activity of strains GW, TOH97, and TOH144 were low and not significantly different from each other. Thus, inactivation of CspA did not appear to have a detectable effect on C5a activity, as evaluated in this functional assay. C5a protease activity only correlated with the presence of functional ScpB. The above results, taken together, are consistent with the conclusion that CspA does not function as a C5a protease.

**Evaluation of GBS phenotypic traits.** We evaluated the expression of several GBS phenotypic traits, some of which are known GBS virulence determinants. The cspa mutant expressed α-hemolysin, CAMP factor, hippuricase, and hyaluronidase similarly to wild-type GBS (data not shown). These strains also expressed equivalent amounts of type III capsule as measured by competitive ELISA (72.7 ± 15.8 µg/mg for COH1 and 82.9 ± 26.3 µg/mg dry weight for TOH121). Invasion of A549 epithelial cell monolayers was 1.2% ± 0.3% for COH1 and 1.3% ± 0.2% for TOH121, expressed as a percentage of the number of total input bacteria invading the A549 cells. Many of the proteases that are homologous to CspA are important for bacterial growth. For example, caseinases from lactic acid bacteria participate in the degradation of extracellular casein before utilization of the casein peptides as a nutritional source. Because GBS is auxotrophic for multiple amino acids, it must rely on exogenous amino acids. Thus, we compared the growth characteristics of the cspa mutant to the wild-type strain in different growth media. Growth of the strains was comparable in all media that were tested, including RPMI plus 5% Casamino acids and THB (data not shown). Growth in human plasma was compared, and the strains exhibited doubling times of 0.6 hours and 0.52 hours for COH1 and TOH121, respectively. This suggests that CspA does not play a role in nutritional scavenging, at least under the experimental conditions tested.

**Caseinase activity of GBS.** Because of the strong similarity of CspA to PrtS, which functions as a caseinase, we tested whole cells of COH1 and TOH121 for casein degradation. Whole GBS cells were incubated with casein, the mixture was centrifuged, SDS-PAGE was performed on the supernatant, and the amount of intact casein was measured by quantitation of Coomassie-stained gels. Very little casein degradation was observed under the experimental conditions employed, and experiments with the two strains yielded similar results (Figure 5).

**Figure 6**

Fibrinogen degradation assay. GBS strains COH1 (wild type) and TOH121 (cspa−) were grown to stationary phase in THB. Cells were washed once in an equivalent volume of PBS and concentrated 20-fold. Fibrinogen was then added to a concentration of 0.61 µg/µl. The suspension was incubated with slow rotation overnight (16 hours) at 37°C, bacteria were removed by centrifugation, and the supernatant, containing 3 µg of total fibrinogen, was analyzed by SDS-PAGE as follows: lane 1 shows COH1 after 0 hours of incubation; lane 2, TOH121 after 0 hours incubation; lane 3, COH1 after 16 hours of incubation; and lane 4, TOH121 after 16 hours of incubation. The arrow at the left of the figure denotes the migration position of the minor species of the α fragment of fibrinogen that is proteolyzed by CspA. Migration of molecular mass markers (in kDa) is indicated on the right.
The cspA gene is required for cleavage of human fibrinogen.

We hypothesized that CspA, as a putative surface-localized protein, proteolyses a host factor. Therefore, to test the ability of CspA to function as a protease, we compared the ability of the cspA mutant and the wild-type strain to degrade a variety of host proteins. We tested purified human fibronectin, purified complement component C3, and purified human fibrinogen. To evaluate degradation, whole bacteria and/or mutanolysin-extracted surface proteins of COH1 and TOH121 were incubated with the test substrate, and degradation was assessed by evaluation of Coomassie-stained SDS-PAGE gels or by Western blotting. No difference in the cleavage of the test substrates was observed for all substrates except fibrinogen when the mutant and wild-type strains were compared (data not shown).

Human fibrinogen was cleaved by the wild-type strain but was not cleaved by the cspA mutant TOH121. Fibrinogen is a dimer of nonidentical subunits — α, β, and γ — that are covalently linked together by disulfide bonds (42). For fibrinogen isolated from fresh human plasma, we found that SDS-PAGE resolves the α subunit of fibrinogen into a doublet. We excised both putative α bands from an SDS-PAGE gel and confirmed their identity by MALDI-TOF MS. Peptides matching the sequence of the α fragment of human fibrinogen were obtained from both putative α bands; 28% of the fibrinogen sequence was covered by the tryptic peptides that were obtained. The lower band of the α doublet is believed to be an in vivo cleavage product in which the C-terminal 27 amino acids are removed (43). When the wild-type and CspA mutants were compared for fibrinogen cleavage, SDS-PAGE revealed that COH1 cleaved the minor species (corresponding to the lower band of the doublet) of the α subunit, whereas the mutant did not cleave fibrinogen under the conditions used (Figure 6). We also noticed that when the mutant and wild-type were incubated with fibrinogen overnight, the wild-type strain formed macroscopic aggregates that were much more prominent in comparison with the mutant strain. These aggregates were not observable in controls in which the wild-type strain was incubated in PBS alone.

CspA is essential for virulence in a neonatal rat model. Extracellular proteases have been implicated in virulence both in Gram-negative and Gram-positive bacteria. To determine if CspA contributes to virulence, we compared the LD50 of TOH121 (cspA) to the isogenic wild type strain, COH1. A series of 10-fold dilutions of each strain were introduced by intraperitoneal injection into neonatal rats (24–48 hours old). Five separate lethality experiments were performed. The mean LD50 values were $2.9 \times 10^4$ and $2.9 \times 10^4$ CFU per animal for COH1 and TOH121, respectively ($P < 0.0431$ by the Wilcoxon matched-pair signed-ranks test). These results suggest that mutation of cspA significantly impairs the virulence of GBS in the neonatal sepsis model.

CspA promotes evasion of opsonophagocytosis. We hypothesized that CspA may allow GBS to avoid innate immune clearance in the nonimmune host, perhaps by a novel mechanism, since capsular polysaccharide expression was not affected by the cspA mutation (see above). Opsonophagocytosis is an important mechanism for bacterial clearance, and neutrophils play a major role in the elimination of GBS from the bloodstream (44). We hypothesized that the attenuated virulence of TOH121 may be a consequence of increased susceptibility to phagocytic clearance as compared with COH1. Fresh PMNs were isolated from human donors, and pooled human serum was used as a source of complement and was preabsorbed with COH1 to remove antibodies directed against the bacteria. The assay was repeated four times, and the results of a representative experiment are shown in Figure 5. COH1-13 (16), which is an unencapsulated mutant of COH1 known to be very susceptible to PMN killing, was included as a control. The growth index (GI) of each strain was calculated as the output CFU per milliliter divided by the input CFU per milliliter. COH1 growth during the assay corresponded to $10^3$ and $2.9 \times 10^4$ (Figure 7). In contrast, the negative control strain, COH1-13, was markedly killed by human PMNs during the 1-hour incubation (GI = 0.04). TOH121 exhibited a sensitivity that was intermediate between COH1 and COH1-13 (GI = 0.81). All three strains grew in the presence of heat-inactivated sera and in the absence of PMNs, yielding GIs between 2 and 4 (Figure 7 and data not shown). We also averaged the results of the four experiments and expressed the results as a ratio of the GI of the mutant to the GI of the wild type, which was 0.46 ($P < 0.001$ by Student’s t test). These findings suggest that CspA promotes resistance...
CspA appears to be a novel member of the CEP family of subtilase-like proteases on the basis of its functional properties also. Most of the CEP family members that have been studied are from dairy lactic acid bacteria and function in the acquisition of nutrients through degradation of casein (36). In fact, the CEP family member that CspA shows the highest overall similarity to is PrtS from *S. thermophilus* (12), which catalyzes casein degradation. However, the inactivation of CspA did not affect the ability of GBS to degrade casein in vitro; in fact, GBS did not exhibit substantial caseinase activity under the conditions that we tested. We also considered that CspA may function to degrade other proteins for the purpose of nutrient acquisition. However, we did not detect a difference in growth rates in human plasma when the wild-type strain and the cspA mutant were compared in vitro (doubling times of 0.6 hours and 0.52 hours for COH1 and TOH121, respectively). The findings suggest that CspA does not perform a function homologous to the caseinases (36) and does not play an essential role in nutrient acquisition, at least under the conditions that we tested.

The finding that whole cells of TOH121 (cspA) cannot cleave fibrinogen strongly suggests that cspA encodes a protein with active proteolytic activity. Although it is a formal possibility that the cspA gene is simply necessary for the function of a distinct protease that actually cleaves fibrinogen, the simplest hypothesis to explain our data is that cspA encodes the fibrinogen-degrading protease. It is intriguing that CspA degrades the lower α band of fibrinogen, which corresponds to fibrinogen with the C-terminal 29 amino acids removed (43). Many proteases, including plasmin, will cleave the C terminus of fibrinogen (43), but it appears that CspA acts on the form of fibrinogen that has already been cleaved at the C terminus of the α chain. The significance of this observation is not currently known; however, CspA may proteolyse fibrinogen at a position distinct from the positions acted on by other bacterial proteases.

Another bacterial protease that degrades fibrinogen is the SpeB protease of GAS. SpeB also cleaves fibrinogen, but in a manner that is rather different from what we observed for CspA activity. SpeB degrades the α band of fibrinogen, which leads to the nearly complete disappearance of the α band on SDS-PAGE gels (52). In contrast, CspA does not degrade the major species of the α chain, and its cleavage of the minor α species yields bands that differ in size from the major band by less than 10 kDa. These results suggest that CspA may cleave fibrinogen more specifically than SpeB; additionally, SpeB is a cysteine protease, whereas CspA is a serine protease.

The connection between the attenuated opsonophagocytosis, the reduced virulence of TOH121, and the fibrinolytic activity of CspA is not yet clear. However, the sensitivity of TOH121 to opsonophagocytic killing may reflect the inability of TOH121 to cleave fibrinogen. GBS may be exploiting the adhesive properties of a fibrin-like substance by cleaving host fibrinogen in a manner sim-
ilar to thrombin. Fibrin is formed from fibrinogen and is the key structural element of blood clots as well as a component of the extracellular matrix. In blood, the proteolysis of the N termini of the α and β chains of fibrinogen leads to the polymerization of cleaved fibrinogen, forming fibrin, which, as the structural component of blood clots, is highly adhesive. If CspA cleaves the α chain of fibrinogen at the N terminus, it could potentially expose the charged regions present on fibrinogen that are responsible for polymerization of fibrinogen and may promote the aggregation of GBS or the coating of GBS with fibrin. We have observed that when the wild-type strain (COH1) is incubated with fibrinogen, aggregates are formed and can be observed as macroscopic particles, whereas in the TOH121 (csp A) mutant strain, aggregation is much less prominent. These aggregates may represent GBS complexed with a fibrinogen-derived cleavage product.

One explanation for the reduced opsonophagocytosis of TOH121 is that wild-type GBS (csp A) is coated with a fibrin-like substance and this substance reduces the access of opsonins or neutrophils to GBS. The other possibility is that the cellular aggregation is induced by fibrinogen cleavage; aggregated GBS may be protected from host-defense mechanisms. A third possibility is that the reduced opsonophagocytosis of GBS is not a consequence of CspA-cleaved fibrinogen. For example, it is possible that CspA proteolyses a protein distinct from fibrinogen and that degradation of this protein is responsible for the attenuated opsonophagocytosis of TOH121. Such a protein could even be bacterial in nature; the SpeB protease of GAS is known to cleave bacterial surface proteins (52). We tested for degradation of the GBS αC protein and found that the csp A gene was not required for cleavage of this surface-localized protein (data not shown). Further studies will be needed in order to link the defect in virulence to the attenuated opsonophagocytosis and fibrinogen cleavage.

We attempted to complement the csp A mutant in order to verify that that decreased opsonophagocytosis, virulence, and defect in fibrinogen cleavage were linked. However, we were unable to subclone csp A to a plasmid; attempts at cloning on both high- and low-copy plasmids failed. We are currently attempting to replace the erm'/ cassette of TOH121 with the wild-type gene in order to demonstrate the causality of the csp A::erm' mutation.

Gram-positive bacteria are important human pathogens; however, much remains to be understood concerning how Gram-positive bacteria evade opsonophagocytosis. To our knowledge, this study represents the first report of a GBS protease that functions in the evasion of opsonophagocytosis. In summary, we have identified a novel GBS protease with extensive homology to the C5a-asces of GAS and GBS as well as to other members of the subtilase family of serine proteases. All GBS serotypes that were examined bore the csp A gene. Mutation of csp A attenuated GBS virulence in a neonatal rat sepsis model and decreased resistance to phagocytosis. The protease is necessary for the cleavage of human fibrinogen. Understanding the contribution of this novel serine protease–like gene to the pathogenesis of GBS disease will broaden our understanding of how this significant pathogen causes severe infections of the newborn infant.

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