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Streptococcus pneumoniae endopeptidase O (PepO): A Multifunctional Plasminogen and Fibronectin Binding Protein, Facilitating Evasion of Innate Immunity and Invasion of Host Cells

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Running title: *PepO a novel plasminogen and fibronectin binding protein*

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Keywords: Plasminogen; fibronectin; invasion; pneumococci

Background: Pneumococci have developed multiple strategies to infect the host.
Results: PepO is ubiquitously expressed pneumococcal protein that interacts with host proteins and facilitates host cell invasion and evasion of innate immunity.
Conclusion: PepO is a plasminogen and fibronectin binding pneumococcal invasin.
Significance: Understanding the mechanism of pneumococcal interaction with host aids designing better therapeutical strategies and gaining control over the pathogen.

SUMMARY

***Streptococcus pneumoniae* infections remain a major cause of morbidity and mortality worldwide. Therefore a detailed understanding and characterization of the mechanism of host cell colonization and dissemination is critical in order to gain control over this versatile pathogen. Here we identified a novel 72 kDa pneumococcal protein endopeptidase O (PepO), as a plasminogen and fibronectin binding protein. Using a collection of clinical isolates, representing different serotypes, we found PepO to be ubiquitously present both at the gene and at the protein level. In addition, PepO protein was secreted in a growth-phase dependent manner to the culture supernatants of the pneumococcal isolates. Recombinant PepO bound human plasminogen and fibronectin in a dose-dependent manner and plasminogen did not compete with fibronectin for binding PepO. PepO bound plasminogen via lysine residues and the interaction was influenced by ionic strength. Moreover, upon activation of PepO bound plasminogen by urokinase-type plasminogen activator, generated plasmin cleaved complement protein C3b thus assisting in complement control. Furthermore, direct binding assays demonstrated the interaction of PepO with epithelial and endothelial cells that in turn blocked pneumococcal adherence. Moreover, a *pepO*-mutant strain showed impaired adherence to and invasion of host cells compared to their isogenic wild-type strains. Taken together, the results demonstrated that PepO is ubiquitously expressed plasminogen and fibronectin binding protein, which plays role in pneumococcal invasion of host cells and aids in immune evasion.**

The Gram-positive bacterium *Streptococcus pneumoniae* (the pneumococcus) is a commensal that asymptomatically colonizes the upper respiratory tract. However, alterations within host-pathogen homeostasis result in the ability of pneumococci to gain access to the normally sterile parts of the airways and cause infections. These include mild local infections such as otitis media and sinusitis or life threatening invasive diseases, including lobar pneumonia, sepsis and meningitis (1). The pneumococcus is the prime cause of community-acquired pneumonia in adults and

accounts for two-thirds of all cases of bacteraemic pneumonia (2). In addition, pneumococcal septicemia is a major cause of infant mortality in developing countries, amongst children under the age of 5 years (3).
Pneumococci utilize multiple mechanisms for colonization of the respiratory tract, transcytosis through host cells, dissemination into the blood stream and in evasion of the host immune attack (4-6). Surface proteins like choline-binding proteins, LPxTG motif containing proteins and lipoproteins (7-9), have been described as ligands for cellular receptors and binding molecules interacting with various components of the extracellular matrix (ECM)[#] or serum proteins of the host. For example, pneumococcal surface protein C (PspC) is a multifunctional choline-binding protein and a major virulence factor of pneumococci that interacts directly and in a human specific manner with the polymeric immunoglobulin receptor, and mediates pneumococcal adherence and invasion of host cells (10-12). In addition, lipoproteins such as ABC metal permease PsaA (13), a pathogenicity-island encoded protein PsrP (14,15) and pili, expressed by a subclass of pneumococci (16,17) have been suggested to have adhesive functions. Furthermore, number of pneumococcal proteins interacts with fibronectin or plasminogen, a strategy commonly employed by many invading pathogens to colonize or disseminate within the host (18,19). These include Pneumococcal adherence and virulence factor (Pav) A, PavB, Plasmin and fibronectin binding protein (Pfb) A as well as PfbB that have been identified as plasminogen and fibronectin binding proteins (20-24).
Fibronectin is a high molecular weight glycoprotein present as an insoluble component in the ECM, on cell surface, and in the basement membrane or as a soluble component in plasma and other body fluids (25). It is highly conserved amongst vertebrates and plays a pivotal role in cell adhesion, growth, differentiation and migration and is an important factor in wound healing and embryonic development (26). Plasminogen, a single chain glycoprotein, is a proenzyme of the serine protease plasmin and plays an important role in fibrinolysis (27), homeostasis (28) and in degradation of ECM (29). In addition to above-mentioned proteins, pneumococci can bind plasminogen by surface displayed enolase and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (30,31). Additionally, interactions with vitronectin, thrombospondin-1 and complement inhibitor Factor H (FH) facilitate bacterial adherence to and invasion of host cells (32-35).
Despite the knowledge and understanding of various virulence factors, the use of antibiotics and the availability of vaccines, the mortality rate due to pneumococcal infection remains alarming. Consequently there is an urgent need to develop new and improved therapies and alternative pneumococcal vaccines to combat pneumococcal diseases. The most promising approach is to develop vaccines based on pneumococcal proteins that contribute to virulence and are common to all serotypes. Therefore, the identification of the essential factor(s) facilitating colonization and subsequent dissemination and the elucidation of the molecular mechanism of pneumococcal-host interactions are important.
While screening the pneumococcal genome for novel proteins that might contribute to virulence, we identified a 72 kDa protein endopeptidase O (PepO), a predicted metallo-endopeptidase that shares homology with M13 peptidase family. M13 peptidase family includes mammalian endopeptidases such as neutral endopeptidase (NEP), involved in regulation of many physiological and pathological processes including various aspects of immune response, and endothelin converting enzyme 1 (ECE1) responsible for processing endothelin-1 into its biologically active form. In this study we identify PepO as a new ubiquitously expressed plasminogen and fibronectin binding protein. We found that PepO protein interacts with human epithelial and endothelial cells and inactivation of the *pepO* gene in *S. pneumoniae* significantly reduced bacterial ability to invade these cells.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions
All pneumococcal strains used in this study, except for NCTC10319 and D39, were isolated from blood of patients suffering from sepsis in the Southwest county of Skåne, Sweden (Table I). For *in vitro* experiments pneumococci were cultured on blood agar plates at 37°C and 5% CO_2, or in Todd-Hewitt-broth (Oxoid) supplemented with 0.5% yeast extract (THY) to a density of 5 x 10^8

CFU/ml (approximately OD_{600} of 0.5). *Escherichia coli* strains DH5α and BL21 (DE3) were cultivated on Luria- Bertani agar or broth.

Generation of pepO-mutant and preparation of recombinant protein

Isogenic mutants that do not express PepO were constructed for the encapsulated D39 strain and for *S. pneumoniae* NCTC10319, which is a low encapsulated strain and thus suitable for cell culture infection experiments as described earlier (32,36). PepO-deficient mutants of D39 and NCTC10319 were generated by replacement of the *pepO* sequence with the spectinomycin gene cassette. Briefly, the full-length *pepO* gene was amplified by PCR from the chromosomal DNA of *S. pneumoniae* D39 with the primers 5- CCATGGCACGTTATCAAGATGATTT -3 and 5- CTCGAGCCAAATAATCACGCGCTC -3, which incorporated flanking *Nco*I and *Xho*I (underlined) restriction sites. The amplified DNA was cloned in pJET1.2 (Fermentas) and later into pET28a for protein expression (Novagen). For generation of a *pepO*-mutant, the inserted *pepO* fragment was digested using *EcoR*V and the spectinomycin gene cassette was blunt-end ligated with the plasmid. The integrity of the antibiotic gene cassette was verified by PCR. The transformation of pneumococci was performed as described previously using competence-stimulating peptide-1 (NordicBiosite) and cultivated in the presence of spectinomycin (50 μg/ml) (37). Transformation of *E. coli* strains with plasmid DNA was carried out with $CaCl_2$-treated competent cells according to a standard protocol.

Proteins and antibodies

Pneumococcal PepO with an N-terminal His_6-tag was expressed from the pET28a vector in E. coli BL21 (DE3) (Stratagene) and purified using a nickel-nitrilotriacetic acid column (GE Healthcare) according to manufacturer's instruction. Polyclonal antiserum against purified PepO was raised in rabbits by routine immunogenic procedures using His_6-tagged-PepO as antigen (Agrisera, Sweden). Purification of rabbit anti-PepO antiserum was performed by affinity chromatography using protein-G sepharose columns (GE Healthcare). Human glu-plasminogen, human-fibronectin and *sheep* anti-human plasminogen antibodies (Abs) were purchased from Haematologic Technologies, while rabbit anti-human fibronectin Abs and peroxidase conjugated swine anti-rabbit IgG and rabbit anti-sheep IgG Abs were purchased from DakoCytomation. Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane was purchased from Sigma, while C3b was from Complement Technology. Proteins were labeled with ^{125}I using the chloramine T method. Normal human serum was prepared from freshly drawn blood obtained from 6 healthy volunteers with informed consent and permission of the ethical board of Lund University. The pooled blood was allowed to clot for 30 min at room temperature (RT) and then incubated for 1 h on ice. After two centrifugations, the serum fraction was frozen in aliquots and stored at -80°C.

Western blot analysis

Purified proteins, bacterial lysates or culture supernatants were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane and submitted to Western blotting using a rabbit polyclonal anti-PepO Abs and a secondary peroxidase conjugated Abs. The polyclonal anti-PepO IgG was used at a dilution of 1:1,000 and the swine anti-rabbit IgG peroxidase conjugate (DakoCytomation) was used at 1:2,000 dilutions and the activity was detected using diaminobenzidine (DAB) (Sigma) and H_2O_2.

Direct binding assays

Microtiter plates (Maxisorb; Nunc) were coated with 50 μl of PepO, plasminogen or fibronectin at a concentration of 5 μg/ml in 75 mM sodium carbonate buffer (pH 9.6) overnight at 4°C. Wells coated with 1% BSA (Applichem) were used as control. Blocking was performed with 250 μl of blocking solution (50 mM Tris HCl (pH 8), 150 mM NaCl, 0.1% Tween-20, 3% fish gelatin (Norland)) for 2 h at RT. PepO, plasminogen or fibronectin were diluted in binding buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, and 50 μg/ml BSA) and incubated with the immobilized plasminogen or fibronectin for PepO, and with immobilized PepO for plasminogen or fibronectin binding, at concentrations indicated in the figures. For

investigation of the effect of ionic strength and the presence of lysine analogue ε-ACA (epsilon-amino caproic acid) (Sigma) on plasminogen-PepO interactions, the binding buffer was supplemented with NaCl to final concentration ranging from 0 to 800 mM or with ε-ACA to final concentration ranging from 0 to 40 mM. The plates were washed with 50 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.1% Tween-20 between each step. Bound PepO, plasminogen and fibronectin were detected with a rabbit anti-PepO, sheep anti-human plasminogen and rabbit anti-human fibronectin polyclonal Abs respectively, followed by a swine anti-rabbit peroxidase conjugate or rabbit anti-sheep peroxidase conjugated Abs (DakoCytomation). The plates were developed with *o*-phenylenediamine (OPD) (DakoCytomation) substrate and H_2O_2, and the absorbance at 490 nm was measured (Varian Cary 50 MPR Microplate Reader).

Binding from serum

Microtiter plates were coated with 50 μl of PepO at a concentration of 5 μg/ml in 75 mM sodium carbonate buffer (pH 9.6) overnight at 4°C. Wells coated with 1% porcine gelatin (Sigma) were used as controls. After incubation with blocking solution for 2 h at RT, human serum diluted in binding buffer was added to the wells at concentrations indicated in figures. After incubation for 1.5 h at RT, bound plasminogen was detected using sheep anti-human plasminogen Abs followed by a rabbit anti-sheep peroxidase conjugated Abs. The plates were developed with OPD substrate and H_2O_2 and the absorbance at 490 nm was measured.

Biacore Analysis

To determine the kinetics of PepO binding to fibronectin or plasminogen, surface plasmon resonance (SPR) analysis was performed using Biacore 2000. Fibronectin or plasminogen were diluted to 50 μg/ml in 10 mM sodium acetate (pH 4.0) and immobilized on the surface of a CM5 sensor chip to reach 2466 and 2800 response units (RU), respectively. All experiments were performed at a continuous flow rate of 30 μl/min using Biacore buffer (150 mM NaCl, 10 mM Hepes, 0.002% Tween-20, pH 7.4). The analyte, PepO, was injected in a concentration gradient as indicated in the figure followed by two consecutive injections of 2M NaCl, 100 mM HCl and 0.05% SDS for regeneration. The obtained sensorgrams were analyzed using Bio-evaluation software 3.0 using 1:1 Langmuir binding model of interaction with drifting baseline.

Plasminogen activation assay

PepO (5 μg/well) coated plate was incubated with plasminogen (1 μg) in binding buffer for 1.5 h at RT. After extensive washing, 10 U/well of the activator uPa (urokinase-type plasminogen activator) (Sigma) was added and the activity of newly generated plasmin was assayed with chromogenic substrate S-2251 (H-D-Valyl-L-leucyl-L-lysine-p-Nitroaniline dihydrochlo-ride) (Chromogenix). The plate was incubated at 37°C, and cleavage of the chromogenic substrate was followed for the time period indicated in the figure by measuring the absorbance at 405 nm.

C3b, Fibrinogen and extracellular matrix protein degradation

Microtiter plates were coated with 50 μl of PepO (5 μg/well) in PBS overnight at 4°C. Blocking was performed with 250 μl of blocking solution for 2 h at room temperature. Plasminogen (5 μg/well) diluted in binding buffer was incubated with the immobilized PepO for 1.5 h at RT. After washing, fibrinogen (5 μg/well), ^{125}I-labeleld C3b, fibronectin or laminin (100 kcpm) together with uPa (10 U/well) were added and incubated at 37°C for indicated time points. Fibronectin and laminin degradation was followed for 18h and stopped by addition of reducing SDS-PAGE sample buffer and boiling at 95°C for 5 min. Thereafter the samples were separated by SDS-PAGE. Degradation of fibrinogen was evaluated by subjecting the sample to Western blot using rabbit antiserum to human fibrinogen, followed by swine anti-rabbit IgG peroxidase conjugate. For C3b degradation, positive and negative control reactions were prepared with Factor H and with or without Factor I, respectively. Degradation products of C3b, fibronectin and laminin were visualized using a PhosphoImager (Fuji).

Pneumococcal binding to immobilized fibronectin

Microtiter plates were coated with 50 μl of

fibronectin at a concentration of 5 μg/ml in PBS overnight at 4°C. Blocking was performed with 250 μl of blocking solution for 2 h at RT. Labeling of the bacteria was performed by incubating 2 x 10^9 cfu pneumococci with fluorescein-isothiocyanate (FITC) (1 mg/ml) in 75 mM sodium carbonate buffer (pH 9.6) for 1 h at 37°C. After extensive washing, the FITC-labeled pneumococci (1 x 10^8 cfu/ml) were added to the wells and incubated for 1 h at 37°C for binding. Fluorescence was measured at 485 nm/535 nm (excitation/emission) using a Victor^2 1420 multilabel counter (Wallac). Measurements were done after three washing steps with 50 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.1% Tween-20.

Bacteria ELISA

Pneumococcal strains D39, NCTC10319 and their corresponding isogenic *pepO*-mutant strains were cultured overnight on blood agar plates, washed in PBS and adjusted to 10^8 CFU/ml. Microtiter plates were coated with 50 μl of bacterial suspension and incubated overnight at 37°C. After incubation with blocking solution for 2 h at RT, plasminogen diluted in binding buffer was added to the wells at concentrations indicated in the figure. After incubation for 1.5 h at RT bound plasminogen was detected using sheep anti-human plasminogen Abs followed by a rabbit anti-sheep peroxidase conjugated Abs. The plates were developed with OPD substrate and H_2O_2 and the absorbance at 490 nm was measured.

Cell lines and culture conditions

Epithelial cells were cultivated as described previously (32). Briefly, human A549 cells (lung alveolar epithelial cells, type II pneumocytes, ATCC catalog no. CCL 185) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) (PAA Laboratories) supplemented with 10% of heat inactivated FCS (Invitrogen), 2 mM glutamine (PAA Laboratories), penicillin G (100 Units/ml) and streptomycin (0.1 mg/ml) (both from Hyclone) at 37°C under 5% CO_2 atmosphere. Human umbilical vein endothelial cells (HUVECs) were obtained from Invitrogen and cultivated in M200 media supplemented with low serum growth supplement (Invitrogen). The cells were used for experiments between passages 1 and 5.

Flow cytometric analysis of PepO binding to epithelial cells

Binding of recombinant PepO to A549 epithelial cells and HUVECs was measured using flow cytometry. A549 cells were seeded at a density of 1 x 10^5 cells per well while HUVEC at 5 x 10^4 cell per well in plain medium on 6-well tissue culture plates (Nunc) and cultivated for 48 h. Confluent monolayers were washed thoroughly and incubated with recombinant PepO protein at the concentration mentioned in figures in culture medium for 3 h at 4°C. Following the incubation, the cells were thoroughly washed with cold PBS + 0.05% FCS and PepO bound to the epithelial cells was stained by incubating with polyclonal rabbit anti-PepO Abs at a dilution of 1:1000 in PBS + 0.05% FCS for 1 h on ice. Thereafter the cells were carefully washed and bound Abs were detected with an Alexa Fluor 488 (green) labeled goat anti-rabbit Ig (Invitrogen) for 1h on ice. Finally after washing, the cells were fixed using 1% paraformaldehyde (PFA; Sigma) and scrapped from the plate. The flow cytometry analysis was performed using CyFlow space (Partec) to detect the binding of PepO. Cells were detected using log-forward and log-side scatter dot-plot, and a gating region was set to exclude debris and larger aggregates of cells. Fluorescence from 15,000 cells/events were analyzed using log-scale amplifications. The geometric mean fluorescence intensity (GMFI) was recorded as a measure for binding activity.

Fluorescence microscopy

Recombinant PepO attached to host cells was stained using rabbit polyclonal anti-PepO Abs in combination with a secondary goat anti-rabbit IgG coupled with Alexa Fluor 488 (green) (Invitrogen). Post-incubation of cells with PepO, non-specific binding sites were blocked with 10% FCS and before incubating the cells with PepO Abs (1:100) the cell layer was thoroughly washed with PBS. Bound Abs were detected with an Alexa Fluor 488-labeled goat anti-rabbit Ig conjugate (Invitrogen). The glass cover slips were embedded “up side down” in mounting media (DakoCytomation), sealed with nail polish and stored at 4°C. A confocal laser scanning microscope (Zeiss

LSM 510 META) and the appropriate software was used for the image acquisition.

Pneumococcal host cell adherence and invasion assay

A549 cells were seeded at a density of 5 x 10^4 cells per well in plain medium either on 24-well tissue culture plates (Nunc) or on glass cover slips (diameter, 12 mm) when assayed by immunofluorescence and cultivated for 48 h. HUVEC cells were seeded at a density of 3 x 10^4 cells per well. Confluent monolayers were washed thoroughly and infected with pneumococci in DMEM medium supplemented with 1% FCS (infection medium) at 37°C using a multiplicity of infection (MOI) of 25. The infection assays were carried out for 3 h in a total volume of 500 μl after adding the bacteria. Post-infection cells were washed three times with infection media to remove unbound bacteria. The total number of adherent and intracellular recovered bacteria was monitored after detachment and lysis of cells with saponin (Sigma) (1% w/v) and plating the bacteria on blood agar plates. The number of viable intracellular bacteria was quantified by employing the antibiotic protection assay as described (32). Briefly, host cells were infected with pneumococci for 3 h at 37°C under 5% CO_2. Then, the infected cells were washed and the host cells were incubated for 1 h with infection medium containing 100 μg/ml gentamicin (Sigma) and 100 Units/ml penicillin G (Sigma) at 37°C and 5% CO_2 to kill extracellular and non-adherent pneumococci. Invasive and viable pneumococci were recovered from the intracellular compartments of the host cells by a saponin-mediated host cell lysis (1.0% w/v) and the total number of invasive pneumococci was monitored after plating sample aliquots on blood agar plates, followed by colony formation and enumeration. In inhibition experiments, infection assays were carried out in the presence of recombinant PepO protein. Each experiment was repeated at least three times, and results were expressed as mean ± standard deviation (SD).

Whole blood killing assay- Pneumococcal strain D39 and its isogenic *pepO*-mutant strain were grown, washed and resuspended in PBS. Diluted cultures (10 µl) containing 10^3 CFU were mixed with 250 µl of refludan (50 µg/ml) (Pharmion) treated fresh human blood and the mixture was rotated for 3h at 37°C. Viable bacterial counts were determined by plating samples onto blood agar.

RESULTS

PepO is a ubiquitously expressed pneumococcal protein

To determine the presence of the gene encoding for PepO, genomic DNA was extracted from several clinical isolates of pneumococci and subjected to PCR using the *pepO* gene specific primers. The results showed the presence of a 1,893 bp long *pepO* gene in all the tested clinical isolates belonging to different serotypes (Table I) (Fig. 1*A*). Thereafter we tested whether the *pepO* gene was expressed. The pneumococcal cell lysates were separated by SDS-PAGE and transferred onto a PVDF membrane. The presence of PepO protein was detected using polyclonal rabbit anti-PepO Abs. The results showed the presence of a protein band corresponding to 72 kDa band of PepO in all the tested serotypes (Fig. 1 *B*, upper and lower panel). In addition, flow cytometry analysis showed that anti-PepO antiserum bound to the surface of *S. pneumoniae* NCTC10319 strain (Fig. 1*C*). In addition we checked if the *pepO* gene is conserved amongst various pneumococcal strains and other bacterial species. The data clearly indicated that PepO is highly conserved not only within pneumococci but also amongst other streptococcus species (Table II).

Secretion of PepO by Streptococcus pneumoniae

Pneumococci express many non-classical cell surface proteins such as the glycolytic enzymes alpha-enolase and GAPDH, which lack both the LPxTG motif as well as the signal peptide but are surface exposed and secreted by the bacteria (30,31). Therefore, we tested whether PepO could also be secreted by pneumococci. Collections of pneumococcal isolates, representing different capsule serotype (Table I) were cultured in THY medium till mid-logarithmic phase and the culture supernatants were collected. The supernatants were subjected to SDS-PAGE and analysed by Western blotting using polyclonal anti PepO Abs. PepO was detected in the culture supernatant of all the pneumococcal isolates tested (Fig. 2*A*). In

order to analyse growth phase dependent expression of PepO, *S. pneumoniae* strain NCTC10319 (type35A) was cultured till stationary phase and culture supernatants were collected at regular time intervals. Results of immunoblot analysis demonstrated that PepO was secreted by *S. pneumoniae* throughout its growth cycle (Fig. 2*B* and *C*). Further we could estimate that a 1 ml of early mid-logarithmic phase culture of *S. pneumoniae* strain NCTC10319 and D39 contains about 0.088±0.05 µg and 0.025±0.02 µg of PepO protein, respectively. Taken together, our data indicated that PepO is a ubiquitously expressed secreted pneumococcal protein.

PepO binds plasminogen

Microtitre plates coated with recombinant PepO were incubated with increasing concentrations of plasminogen and binding was detected using specific Abs. BSA was used as a negative control. A dose-dependent binding of plasminogen to immobilized PepO was observed (Fig. 3*A*). Similarly in a reverse setting, a dose-dependent binding of PepO to plasminogen was observed (Fig. 3*B*). Further, SPR was used to analyse the interaction between PepO and plasminogen (Fig. 3*C*). Once again a concentration dependent binding of PepO to immobilized plasminogen was detected (K_D= 92 nM). Representative sensorgrams for the interaction are shown and the calculated kinetic values are presented in Table III. Taken together these data indicated that PepO binds plasminogen with high affinity.

To determine whether the interaction between plasminogen and PepO is hydrophobic or ionic in character, the binding assay was conducted in the presence of varying NaCl concentrations. Binding of plasminogen to PepO decreased with increasing NaCl concentrations (Fig 3*D*). At a NaCl concentration of 800 mM the plasminogen binding to PepO was reduced by 40% as compared to binding at the physiological concentration of 150 mM NaCl. Furthermore, to analyse whether lysine residues are critical for binding of plasminogen to PepO, the effect of ε-ACA, a lysine analogue, was investigated. In the presence of increasing amounts of ε-ACA as lysine analogue, binding of plasminogen was significantly reduced (Fig 3*E*). Thus, binding of plasminogen to *S. pneumoniae* PepO is weakly influenced by ionic strength and it is mediated by lysine residues.

To corroborate the above results we analyzed the recruitment of plasminogen via PepO from human serum. Microtitre plates coated with PepO were incubated with several dilutions of human serum in binding buffer and binding of plasminogen was detected with specific Abs. A dose-dependent increase in binding of plasminogen, proportional to the serum concentration was obtained (Fig. 3*F*).

PepO binds fibronectin

To test binding of PepO to fibronectin, microtitre plates coated with PepO were incubated with increasing concentrations of human plasma fibronectin followed by detection using specific Abs. A dose-dependent binding of soluble fibronectin to immobilized PepO was observed (Fig. 4*A*). Additionally, the binding of pneumococcal PepO to immobilized fibronectin was also detected in reverse settings (Fig. 4*B*). The interaction of fibronectin with immobilized PepO was more readily detectable compared to the binding of PepO to immobilized fibronectin. In addition, the interaction between PepO and fibronectin was analysed by SPR (Fig. 3*C*). A concentration dependent, high-affinity binding of PepO to immobilized fibronectin was detected (K_D= 62 nM; Table III). Representative sensorgrams for the interaction are shown. Taken together, the data confirms that PepO interacts with fibronectin.

Plasminogen does not compete with fibronectin for binding to PepO

To address the question whether plasminogen and fibronectin bind simultaneously to PepO or if they compete with each other for the binding sites on PepO, a constant concentration of plasminogen (5 μg/ml) was added together with increasing concentrations of fibronectin to microtitre plates coated with PepO. The bound plasminogen and fibronectin were detected using specific Abs. Both plasminogen and fibronectin bound to immobilized PepO and the binding of plasminogen was not affected by the presence of increasing concentrations of fibronectin (Fig. 5*A*). However, in the reverse setting, the presence of constant concentration of fibronectin (5 μg/ml) together with increasing concentrations of plasminogen, the binding of fibronectin was slightly affected (Fig. 5*B*). At

a plasminogen concentration of 20 μg/ml the fibronectin (5 μg/ml) binding to PepO was reduced by 37% as compared to binding in the absence of plasminogen. Taken together, the data suggested that the two proteins do not compete with each other for binding to PepO indicating that plasminogen and fibronectin can bind simultaneously and to non-overlapping binding sites on PepO. However, the presence of excess of plasminogen may compete with fibronectin for binding to PepO.

Plasminogen bound to PepO is converted to functionally active plasmin that cleaves the complement protein C3b and ECM components

Plasminogen interacts with complement protein C3 and C5 and upon activation to plasmin, it inactivates complement at the level of C3 and C5 thereby regulating the complement cascade (38). In accordance, activated plasminogen bound to *Haemophilus influenzae* Protein E has been shown to cleave C3b thereby facilitating complement evasion (39). We therefore tested whether plasminogen bound to PepO could be readily activated by its activator uPa and converted to the active protease plasmin. Thus, PepO bound plasminogen was incubated with uPa and generated plasmin activity was evaluated by incubating with the chromogenic substrate S-2251. A time dependent cleavage of the substrate by the generated plasmin was observed (Fig 6*A*). Interestingly, no cleavage of the substrate was observed when the plasminogen was incubated with PepO without addition of uPa or in the presence of only uPa, demonstrating the specific protease activity of the generated plasmin.

In addition, we tested the ability of plasminogen bound to PepO to cleave its natural substrate fibrinogen. To this end, plasminogen bound to immobilized PepO was incubated with uPa and substrate fibrinogen. At indicated time points sample aliquots were taken out and separated by SDS-PAGE and Western blotting was performed to detect fibrinogen fragments. A time dependent cleavage of fibrinogen by the generated plasmin was observed (Fig. 6*B*).

Further, we assayed whether PepO bound plasminogen can also affect complement. For this, plasminogen bound to immobilized PepO was incubated with uPa and ^{125}I-labeled C3b and following the incubation, samples were separated by SDS-PAGE and C3b cleavage products were analysed. A time dependent cleavage of C3b by activated plasminogen (plasmin) was observed (Fig 6*C*). Following the incubation two major bands of about 40 and 30 kDa were obtained. Importantly, no C3b cleavage was observed upon incubation with uPa (Fig. 6*C*), suggesting that the PepO bound plasminogen, cleaved C3b when activated with uPa.

In addition to its ability to cleave fibrin clot and C3b, protease plasmin also acts on ECM components. Therefore, we tested whether plasminogen bound to PepO upon activation could also cleave ECM components such as fibronectin and laminin. Compared to the cleavage of fibronectin by protease plasmin (Fig. 6*D*), which was readily observed, laminin cleavage was present but weak (Fig. 6*E*) after 18h of incubation. Taken together, plasminogen bound to PepO is readily activated to the active protease plasmin.

Role of PepO in pneumococcal binding to fibronectin and plasminogen

To elucidate the role of PepO in pneumococcal binding to fibronectin, *pepO*-mutants of pneumococci were generated by insertional inactivation. The expression of PepO in wild-type strain D39 and NCTC10319 and their respective isogenic mutants (Δ*pepO*) was analysed in the whole cell lysates (Fig. 7*A*) and in the culture supernatants (Fig. 7*B*). The immunoblots revealed a successful disruption of PepO in the mutants (Fig. 7 *A* and *B*). Phosphoglycerate kinase (PGK) was used as a control (Fig. 7 *A* and *B*) (40). A binding assay with FITC-labelled *pepO*-mutants in strain D39 and NCTC10319 background indicated that while PepO of D39 strain contributes significantly to pneumococcal binding to immobilized fibronectin no inhibition was observed with the NCTC10319 *pepO*-mutant (Fig. 7*C*). Compared to their respective wild-type strains, the *pepO*-mutants in D39 background showed 27.2 ± 6.9% binding while the NCTC10319 *pepO*-mutant showed a 74.6 ± 26.4% binding to immobilized fibronectin (Fig. 7*C*). The data suggested a strain-dependent interaction of PepO with fibronectin. Similarly, binding of plasminogen to the wild-type and corresponding *pepO*-mutant strain was analysed by ELISA. A dose dependent binding of plasminogen to the wild-type D39 and NCTC10319 strain was

observed. Moreover, a significant reduction in the plasminogen binding to the *pepO*-mutant in both D39 and NCTC10319 background was observed at a plasminogen concentration of 10 μg/ml. Although a minor reduction in plasminogen binding was observed at other concentrations, it was not significant.

Recombinant PepO protein interacts with host epithelial cells

We found that recombinant PepO protein bound to epithelial cells. As shown in figure 8*A*, flow cytometric analysis revealed a dose dependent binding of recombinant PepO with A549 lung epithelial cells. To corroborate these data, immunofluorescence staining of PepO bound to the host cells was performed and confocal images were taken. Figure 8*B* demonstrates the binding of PepO (Alexa-488 stained, green) with A549 cells.

PepO mediates pneumococcal adherence and invasion of host epithelial cells

In order to analyse the role of PepO in pneumococcal adherence and invasion of host cells, cell culture infection experiments were performed. The A549 human lung epithelial cells A549 were infected for 3h with wild-type NCTC10319 or D39 strains and their respective isogenic *pepO*-mutant (Δ*pepO*). Although, wild-type D39 bacteria showed significant levels of adherence and invasion into these host cells, PepO-deficient bacteria showed a drastic reduction in adherence and invasion (Fig. 8*C* and *D*). Surprisingly, the disruption of the gene encoding PepO in NCTC10319 background had no effect on the adhesive capacity of the bacteria compared to its wild-type strain (Fig 8*C*), whereas compared to its wild-type NCTC10319 strain, the *pepO*-mutant showed a reduction of 30% in the number recovered intracellular pneumococci (Fig. 8*D*). Due to the presence of thick capsule in *S. pneumoniae* D39, the number of adhered and recovered D39 (28±4 x 10^2 CFU/well) was relatively low compared to the NCTC10319 strain (12.57±1.96 x 10^4 CFU/well), nevertheless a clear effect was observed for D39 strain. Since PepO directly interacts with host cells, the ability of recombinant PepO in blocking pneumococcal adherence was investigated. The A549 epithelial cells pre-treated with PepO protein were infected with NCTC10319 and D39 bacteria. The ability of the pneumococci to adhere to the epithelial cells was reduced by 48 and 44% for NCTC10319 and D39 background, respectively (Fig. 8*E*). Taken together, the data suggested that PepO, in a strain dependent manner, facilitates pneumococcal attachment and internalization of host epithelial cell

Pneumococcal adherence and invasion of endothelial cells is facilitated by PepO

Our data demonstrated that PepO interacts with host epithelial cells and facilitates pneumococcal strain dependent adherence and invasion of host cells. Therefore, to investigate if a similar mechanism is also relevant for endothelial cells, firstly binding of recombinant PepO protein to HUVECs was assayed. Similar to A549 epithelial cells (Fig 8*A*), flow cytometric analysis revealed a dose dependent binding of PepO protein to HUVECs (Fig. 9*A*). To assess the role of PepO in pneumococcal adherence and invasion, HUVECs were infected for 3h with *S. pneumoniae* D39 strain and its isogenic *pepO*-mutant. Although wild-type bacteria showed significant levels of adherence to and invasion into endothelial cells, PepO-deficient bacteria demonstrated a significant reduction of 97 and 99% in adherence and invasion, respectively (Fig. 9 *B* and *C*). In addition, we explored the ability to recombinant PepO protein to block pneumococcal adherence to HUVECs. The pre-treatment of HUVECs with recombinant PepO resulted in 43% reduction in the adherence of D39 strain (Fig. 9*B*). In conclusion, our data from the cell culture infection assays indicated that PepO modulates pneumococcal attachment and internalization by host cells.

Influence of PepO on bactericidal activity of human blood

Bactericidal assay was performed to investigate the function of PepO during systemic infections. Approximately 10^3 CFU of *S. pneumoniae* D39 and its isogenic *pepO*-mutant were incubated separately with 250 μl of human whole blood, and the viability was determined by plating and enumeration of the colonies obtained. The results indicate significant killing of both strains in the whole blood after 3h of incubation. However, compared to the approximately 4% survival of wild-type D39 strain, the *pepO*-mutant strain showed only 0.4% survival efficiency (Fig.

9D). The results indicate the important role of PepO in pneumococcal infections.

DISCUSSION

In this study we have identified a 72 kDa pneumococcal protein PepO as a plasminogen and fibronectin binding protein that facilitates pneumococcal invasion of host epithelial cells. PepO is a predicted metallo-endopeptidase that shares homology with the peptidase family M13 proteins. These M13 peptidases are present in a wide range of organisms including mammals and bacteria (41). The M13 family includes neprilysin (neutral endopeptidase), endothelin-converting enzyme I, erythrocyte surface antigen KELL, phosphate-regulating gene with homologies to endopeptidase on the X chromosome, soluble secreted endopeptidase, and damage-induced neuronal endopeptidase (42,43). In mammals, these peptidases have been implicated in processes such as blood-pressure regulation, nervous control of respiration, regulation of the function of neuropeptides in the central nervous system and cardiovascular development. These are typically type-II membrane anchored enzymes, known to activate or inactivate oligopeptide (pro)-hormones. In bacteria they have been implicated in milk protein cleavage (44).

In *Streptococcus pneumoniae*, PepO was found in all the tested strains both at the gene and at protein level, suggesting it to be conserved among the strains. Moreover, within various strains of pneumococci PepO shows 99-100% identity at gene level. Importantly, the expression of PepO was independent of the serotype indicating its ubiquitous expression. Although the gene sequence of PepO lacks both the membrane spanning domains such as the LPxTG motif region, a characteristic feature shared by many surface exposed proteins, and the typical signal sequences, apparently PepO was found on the cell surface and in the culture supernatants, suggesting PepO to be a secreted protein. However, the exact mechanism of PepO secretion or presence into the culture supernatant is still not clear. Interestingly, in pneumococci a number of proteins lacking any signal peptide required for secretion and peptidoglycan-anchoring motifs, are transported to and associated with bacterial surface structures. These include the glycolytic enzyme such as alpha-enolase and GAPDH (30,31) or choline-binding domain containing proteins such as PspC that binds the phosphorylcholine on the pneumococcal cell wall (45).

In this study we found that PepO binds plasminogen in a dose-dependent manner influenced by ionic strength. In addition, a dose-dependent inhibition with the lysine analogue ε-ACA suggests that lysine residues are relevant for the interaction between plasminogen and PepO. This type of interaction is similar to that observed for pneumococcal enolase and plasminogen (46). Similarly interactions of plasminogen with other pathogen proteins such as *H. influenzae* Protein E, *P. aeruginosa* Tuf, *C. albicans* Pra1 and Gmp1 and *B. burgdorferi* CRASP depends on lysine residues and is ionic strength dependent (39,47-50). The specificity of the interaction between PepO and plasminogen was demonstrated in our SPR studies and binding of plasminogen from human serum. Moreover, the pull-down using biotin-labeled plasminogen and co-immunoprecipitation studies using unlabeled plasminogen further confirmed the PepO-plasminogen interactions (data not shown). In addition, we demonstrated that plasminogen bound to PepO was readily activated by uPa to plasmin that not only cleaved its natural substrate fibrinogen but also ECM components such as fibronectin and laminin. In addition it also cleaved C3b thereby mediating complement control. A similar mechanism has been reported for *H. influenzae* Protein E bound plasminogen (39). Interestingly, PepO alone did not cleave plasminogen (data not shown). PepO being a secreted protein may play a role, which can be different from the other cell surface, bound plasminogen-binding proteins. A similar role has been suggested for Skizzle, a secreted protein of group B Streptococcus that binds and enhances urokinase-catalyzed activation of plasminogen (51). Taken together, our data suggests PepO to be an additional component that contributes towards the pneumococcal pathogenesis. Interestingly, PepO did not bind laminin, collagen type II or vitronectin (not shown).

At par with other gram-positive bacteria such as *S. pyogenes* and *Staphylococcus aureus*, pneumococci also express a number of fibronectin-binding proteins such as PfbB, PavB, PavA and PfbA (20-24). Our data indicated that PepO binds fibronectin, in

addition to plasminogen. Both plasminogen and fibronectin are abundant plasma proteins and it seemed highly possible that they may compete with each other for binding to PepO. However, our results demonstrate that binding of plasminogen is not outcompeted by fibronectin indicating that the plasminogen and fibronectin interact with different motifs on pneumococcal PepO. Apparently, the presence of excess of plasminogen seems to compete with fibronectin, suggesting that the binding sites may be present in close proximity resulting in this minor competition.

Our data demonstrate PepO as a secreted protein, which is also present on the bacterial surface and play an important role in pneumococcal survival in whole blood. Even though, PepO appeared to significantly contribute towards the binding of whole bacteria to fibronectin, the ability of PepO to bind host proteins depends on the pneumococcal strain. This was evident by the fact that only the *pepO*-mutant of highly encapsulated D39 serotype-2 strain showed significant reduction in binding to fibronectin compared to the wild-type. Interestingly, the adhesive capacity of the *pepO*-mutant of NCTC10319 type 35A strain was not abrogated compared to its wild-type strain. This could be due to the masking of the PepO effect, by other known and yet unknown surface exposed fibronectin binding proteins in NCTC10319 background, that has relatively thin capsule compared to the D39 strain. Moreover, a similar strain specific effect was demonstrated for PavB, a plasminogen and fibronectin binding protein in pneumococci (21). Additionally these fibronectin binding proteins have been shown to function as adhesins facilitating pneumococcal adherence and invasion of host cells (21,22,24). In accordance, our data indicates that PepO facilitates pneumococcal adhesion and invasion of host cells. A similar effect was observed for both epithelial and endothelial cells suggesting it to be cell-type independent phenomenon. Interestingly, similar to the binding to fibronectin, the role of PepO in bacterial adherence and invasion was also strain dependent, as the inhibition was observed only for the *pepO*-mutant in D39 background. On the contrary, the PepO-deficient NCTC10319 strain showed only 30% reduction in invasion, without affecting the total adherence compared to the wild-type strain. Interestingly, a similar role has been associated to the endopeptidase O from *Porphyromonas gingivalis* that has been demonstrated to play a role in invasion of host epithelial cells but not adherence (52).

In addition, our direct binding studies with soluble PepO protein and host cells showed a dose-dependent binding, suggesting that PepO may interacts directly with a cellular receptor(s). Consistent with this, the presence of soluble PepO in blocking experiments significantly inhibited pneumococcal adherence to host cells. The data thus demonstrate that the PepO interaction with host cells facilitates bacterial colonization and subsequent internalization.

In conclusion, the ubiquitously expressed PepO has the potential to be a new virulence factor facilitating in the pneumococcal infections. Besides having a plasminogen and fibronectin-binding activity, PepO is a novel protein facilitating pneumococcal adherence and internalization of host epithelial and endothelial cells. Moreover, the ability to acquire plasminogen and allow complement control at the C3 level by activated plasmin represents an immune and complement escape mechanism.

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FOOTNOTES

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[#]List of abbreviations:
ECM, extracellular matrix; ε-ACA, epsilon-amino caproic acid; THY, Todd-Hewitt-Yeast extract broth; PavA, pneumococcal adherence and virulence factor A; PavB, pneumococcal adherence and virulence factor B; PepO, endopeptidase O; PfbA, plasmin and fibronectin binding protein A; PfbB, plasmin and fibronectin binding protein B; PspC, pneumococcal surface protein C; SPR, surface plasmon resonance; uPA, urokinase-type plasminogen activator; HUVEC, human umbilical vein endothelial cell; Abs, antibodies; RT, room temperature

FIGURE LEGENDS

FIGURE 1. *Distribution of pepO gene and its expression in various pneumococcal isolates. A,* Agarose gel electrophoresis image showing the amplified product of *pepO* gene (1893 bp) in clinical isolates. *B,* Immunoblots demonstrating the presence of PepO in the bacterial whole cell lysates of pneumococcal isolates. The molecular weight protein marker was used as a reference. *C*, Representative flow cytometry data for the surface presentation of PepO on *S. pneumoniae* NTC10319 serotype 35A.

FIGURE 2. *Secretion of PepO by pneumococci. A,* Immunoblot showing the presence of PepO in the culture supernatant of various isolates of *S. pneumoniae.* Mid logarithmic phase culture-supernatant of pneumococcal isolates were collected and subjected to SDS-PAGE. Presence of PepO protein in the culture supernatant was analysed using rabbit anti-PepO polyclonal Abs. *B and C,* Growth phase dependent secretion of PepO by *S. pneumoniae* strain NCTC10319 serotype 35A. Bacteria were cultured in THY media and culture-supernatant was collected at regular intervals (*B*) and separated by 10% SDS-PAGE (*C*). After blotting, PepO protein was detected using rabbit anti-PepO polyclonal Abs. THY media containing no bacteria was used as control and molecular weight protein marker was used as a reference.

FIGURE 3. *Plasminogen binding to PepO.* Microtiter plates were coated with either (*A*) PepO (5 μg/ml) or (*B*) plasminogen (5 μg/ml) and increasing amounts of plasminogen or PepO was added. Binding was detected using specific polyclonal Abs. BSA was used as negative control. Statistical significance was calculated using two-way Anova and Bonferroni post-test. *C*, Binding of PepO to immobilized plasminogen as analysed by surface plasmon resonance. Increasing concentrations of PepO (22-695 nM) were injected onto plasminogen coated CM5 sensor chip. The amount of PepO associating with the plasminogen was measured in response units. Representative sensorgrams are presented. *D,* Microtiter plates were coated with PepO and the effect of different concentrations of NaCl on binding of plasminogen (5 μg/ml) to PepO was analyzed. Amount of plasminogen bound in the absence of NaCl was set at 100%. Specific polyclonal Abs detected bound Plasminogen. BSA was used as negative control. One-way Anova and Dunnett's post-test were performed to calculate statistical difference compared with the binding at 150 mM NaCl. *E,* The inhibitory effect of the lysine analogue ε-ACA for binding of plasminogen to PepO was evaluated. Specific polyclonal Abs detected bound plasminogen. The signal obtained in the absence of ε-ACA was set to 100% and One-way Anova and Dunnett's post-test was performed to calculate statistical difference compared with the binding in the absence of ε-ACA. *F*, Binding of plasminogen to PepO from human serum was assessed. Microtiter plates were coated with PepO (5 μg/ml) and increasing amounts of serum was added. Bound plasminogen was detected using specific polyclonal Abs. Gelatin was used as negative control. Statistical significance was calculated using two-way Anova test. The data represents the mean ± SD of three independent experiments performed in duplicates. ns: not significant; *p<0.05; **p<0.01; ***p<0.001

FIGURE 4. *PepO binds fibronectin.* Microtiter plates were coated with either (*A*) PepO (5 μg/ml) or (*B*) fibronectin (5 μg/ml) and increasing amounts of fibronectin or PepO was added. Binding was detected using specific polyclonal Abs. BSA was used as negative control. Statistical significance was calculated using two-way Anova and Bonferroni post-test. The data represents the mean ± SD of three independent experiments performed in duplicates. ns: not significant; **p<0.01; ***p<0.001. *C*, Binding of PepO to immobilized fibronectin as analysed by surface plasmon resonance. Increasing concentrations of PepO (22-695 nM) were injected onto fibronectin coated CM5 sensor chip. The amount of PepO associating with the fibronectin was measured in response units. Representative sensorgrams are presented.

FIGURE 5. *Binding of plasminogen and fibronectin to PepO.* PepO was immobilized on microtiter plates. A constant amount of plasminogen (5 μg/ml) together with increasing amounts of fibronectin

(*A*) or constant amount of fibronectin (5 μg/ml) with increasing amounts of plasminogen (*B*) was added. Bound fibronectin and plasminogen were detected using specific Abs. Data presented are from three independent duplicate experiments ± SD. One-way Anova and Dunnett's post-test was used to calculate statistical significance between the binding in the absence and the presence of proteins. *p<0.05; ***p<0.001.

FIGURE 6. *Plasminogen bound to PepO is functionally active. A*, PepO (5 μg/ml) was immobilized on a microtiter plate. After blocking it was incubated with plasminogen (Plg, 1μg/well) in the absence or the presence activator uPa together with the chromogenic substrate S-2251. Measuring the absorbance at 405 nm assessed conversion of the substrate by the generated plasmin. The mean values of three independent experiments and the SDs are indicated. *B*, Degradation of the natural substrate fibrinogen (Fbg) by plasmin(ogen) bound to immobilized PepO. Plasminogen (5 μg/well) was bound to immobilized PepO (5 μg/well) and after washing, uPa (10U/well) together with fibrinogen (5 μg/well) were added. At the indicated time points, sample aliquots were removed, separated by SDS-PAGE, transferred to a membrane, and degradation of fibrinogen was assayed by Western blotting using a rabbit fibrinogen antiserum and a peroxidase-conjugated secondary Abs. Fibrinogen incubated in the absence or presence of uPa alone were used as control. Representative data from three independent experiments are shown. PepO bound plasminogen cleaves complement protein C3b (*C*), ECM components fibronectin (Fbn) (*D*) or Laminin (*E*). Microtiter plates coated with PepO (5 μg/well) were incubated with plasminogen (5 μg/well) followed by addition of uPa (10U/well) and ^{125}I-C3b, fibronectin or laminin (100 kcpm) and incubation at 37°C. Samples were taken at indicated time points for C3b degradation and after 18h incubation for fibronectin and laminin and then separated on a SDS-PAGE. For C3b degradation, the positive control (+ve Ctrl) contained Factor H mixed with Factor I, ^{125}I-labeled C3b, while in the negative control (-ve Ctrl) Factor I was omitted. An arrow marks the cleavage products of C3b. Representative data from three independent experiments are shown.

FIGURE 7. *Effect of PepO-deficiency on pneumococcal binding to fibronectin and plasminogen.* Immunoblot analysis of PepO production in the wild-type (WT) strains D39 and NCTC10319 or their respective isogenic *pepO*-mutants *(ΔpepO*) using (*A*) whole bacterial lysate or (*B*) culture supernatant. Presence of PepO was detected using rabbit anti-PepO polyclonal Abs. Pneumococcal PGK was used as a loading control. *C,* Binding of pneumococci to immobilized fibronectin. Microtiter plates were coated with fibronectin (5 μg/ml) and binding of FITC-labelled *S. pneumoniae* D39, NCTC10319 wild-type or their respective isogenic *pepO*-mutants was assessed. Binding was measured at 485/535 (excitation/emission). The data represents the mean ± SD of three independent experiments performed in duplicates. Statistical significance was calculated using two-way Anova and Bonferroni post-test. ***p<0.001. *D,* Binding of plasminogen to pneumococci. Microtiter plates were coated with 50 μl of 10^8 CFU/ml of *S. pneumoniae* D39, NCTC10319 wild-type or their respective isogenic *pepO*-mutants strains and binding of plasminogen (0, 2.5, 5 and 10 μg/ml) was assessed. Bound plasminogen was detected using specific polyclonal Abs. The data represents the mean ± SD of three independent experiments performed in duplicates. Statistical significance was calculated using one-way Anova and Tukey post-test. *p<0.05; ***p<0.001

FIGURE 8. *PepO mediates pneumococcal adherence and invasion of host epithelial cells. A* and *B*, Binding of recombinant PepO to A549 epithelial cells. Host cells were incubated with increasing amount of recombinant PepO, after washing binding was detected with rabbit anti-PepO Abs followed by Alexa 488-conjugated secondary Abs. Binding of protein was quantified by (*A*) flow cytometry or detected by (*B*) immunofluorescence microscopy. Representative flow cytometry data from three independent experiments are shown. Bar represents 10 μm. *C,* adherence of pneumococci NCTC10319 and D39 and their respective isogenic *pepO*-mutants was determined by counting the cfu per well obtained form sample aliquot plated onto the blood agar plate after 3 h of infection. *D,* invasion and intracellular survival of pneumococci were determined by the antibiotic protection assay. *E*, Recombinant PepO protein inhibits pneumococcal adherence to A549 cells. Epithelial cells were

incubated with recombinant PepO for 30 min prior to infections. Total number of bacteria associated with host cells was determined after removing unbound extracellular bacteria and plating the cells on blood agar plates. The data represents the mean ± SD of three independent experiments performed in duplicates. Statistical significance was calculated using Student's *t*-test. ns: not significant; $**p<0.01$; $***p<0.001$

FIGURE 9. *PepO mediates pneumococcal adherence and invasion of host endothelial cells. A*, Binding of recombinant PepO to endothelial cells (HUVEC). HUVEC were incubated with increasing amount of recombinant PepO and after washing binding was detected with rabbit anti-PepO Abs followed by Alexa 488-conjugated secondary Abs. Representative flow cytometry data from two independent experiments are shown. *B,* adherence of D39 and its isogenic *pepO*-mutants was determined by counting the cfu per well obtained form sample aliquot plated onto the blood agar plate after 3 h of infection. The effect of recombinant PepO protein on wild-type D39 adherence to HUVEC was determined after incubation of cells with PepO (50 μg/ml) for 30 min prior to infections. Total number of bacteria associated with host cells was determined after removing unbound extracellular bacteria and plating the cells on blood agar plates. *C,* invasion and intracellular survival of wild-type and PepO-deficient D39 strain were determined by the antibiotic protection assay. *D*, *S. pneumoniae* D39 and its isogenic *pepO*-mutants strains (10 μl, 1000 CFU) were added to refludan treated whole blood (250 μl) and then gently mixed for 3 h at 37 °C. The bacterial survival was determined by plating onto blood agar plates and determining the number of CFU obtained. The data represents the mean ± SD of three independent experiments performed in duplicates. Statistical significance was calculated using Student's *t*-test. ns: not significant; $*p<0.05$; $***p<0.001$

Table I: Clinical *Streptococcus pneumoniae* isolates used in the present study.

Strains	Serotype	Clinical presentation [a]
S. pneumoniae		
D39	2	-
KR406	3	4
KR407	3	3
KR408	4	3
KR409	6A	3
KR410	6A	2
KR411	23A	4
KR412	7F	3
KR414	8	3
KR415	8	3
KR417	9V	4
KR418	9V	3
KR419	11	3
KR420	14	3
KR421	14	3
KR422	14	1
KR426	19F	4
KR427	19	2
NCTC10319	35A	-

[a] 1= bacteraemia; 2= sepsis; 3= severe sepsis; 4= septic shock

Table II: *pepO* Gene sequence identity in pneumococci and other bacterial species.

Bacterial species	Gene ID	Gene sequence identity (%)
S. pneumoniae R6	spr1491	100
S. pneumoniae P1031	SPP_1666	99
S. pneumoniae Hungary 19A-6	SPH_1756	99
S. pneumoniae JJA	SPJ_1542	99
S. pneumoniae TIGR4	SP_1647	99
S. pneumoniae G54	SPG_1557	99
S. pneumoniae 70585	SP70585_1688	99
S. pneumoniae ST556	MYY_1572	99
S. pneumoniae TCH8431/19A	HMPREF0837_11890	99
S. pneumoniae CGSP14	SPCG_1620	99
S. pneumoniae 670-6B	SP670_1734	99
S. pneumoniae ATCC 700669	SPN23F16490	99
S. pneumoniae gamPNI0373	HMPREF1038_01631	99
S. pneumoniae OXC141	SPNOXC_14460	99
S. pneumoniae AP200	SPAP_1655	99
S. pneumoniae INV104	INV104_13990	99
S. pneumoniae INV200	SPNINV200_14720	99
S. pneumoniae Taiwan19F-14	SPT_1587	99
S. pneumoniae SPNA45	SPNA45_00593	99
S. pseudopneumoniae IS7493	SPPN_08030	97
S. mitis B6	smi_0639	93
S. oralis Uo5	SOR_1505	85
S. gordonii Challis NCTC7868	SGO_1799	72
S. suis 05ZYH33	SSU05_2082	70
S. suis 98HAH33	SSU98_2085	70
S. thermophilus CNRZ1066	str1885	68
S. thermophilus LMG18311	stu1885	68
S. thermophilus LMD-9	STER_1860	68
S. salivarius SK126	STRSA0001_0505	68
S. mutans UA159	SMU_2036	67
S. pyogenes MGAS2096	MGAS2096_Spy1815	66
S. pyogenes MGAS9429	MGAS9429_Spy1793	66
S. pyogenes MGAS10394	M6_Spy1781	66
S. pyogenes MGAS8232	spyM18_2153	66

Table III: Affinity and rate constants for interactions between PepO and plasminogen/fibronectin obtained using SPR analysis.

Interaction	[a]K_D [M]	[b]K_a[1/Ms]	[c]K_d [1/s]	Chi2
PepO-plasminogen				
Experiment 1	8.76e-8	3.29e4	2.88e-3	5.26
Experiment 2	9.7 e-8	3.17e4	3.07e-3	4.27
PepO-fibronectin				
Experiment 1	5.83e-8	4.1e4	2.39e-3	6.16
Experiment 2	6.53e-8	4.01e4	2.61e-3	4.75

[a]K_D – equilibrium dissociation constant; [b]K_a – association rate affinity constant; [c]K_d – dissociation rate affinity constant

Figure 1

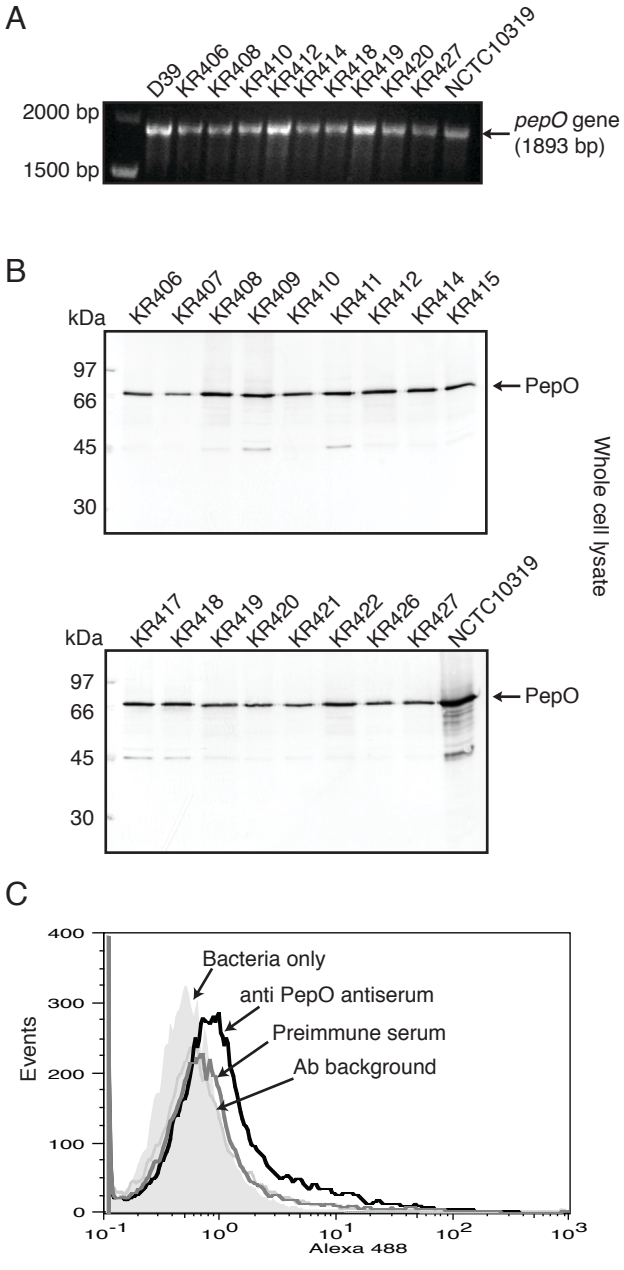

A
D39
KR406
KR408
KR410
KR412
KR414
KR418
KR419
KR420
KR427
NCTC10319
2000 bp
1500 bp
pepO gene
(1893 bp)
B
kDa
KR406
KR407
KR408
KR409
KR410
KR411
KR412
KR414
KR415
97
66
45
30
PepO
Whole cell lysate
kDa
KR417
KR418
KR419
KR420
KR421
KR422
KR426
KR427
NCTC10319
97
66
45
30
PepO
C
Events
400
300
200
100
0
Bacteria only
anti PepO antiserum
Preimmune serum
Ab background
10-1
100
101
102
103
Alexa 488

Figure 2

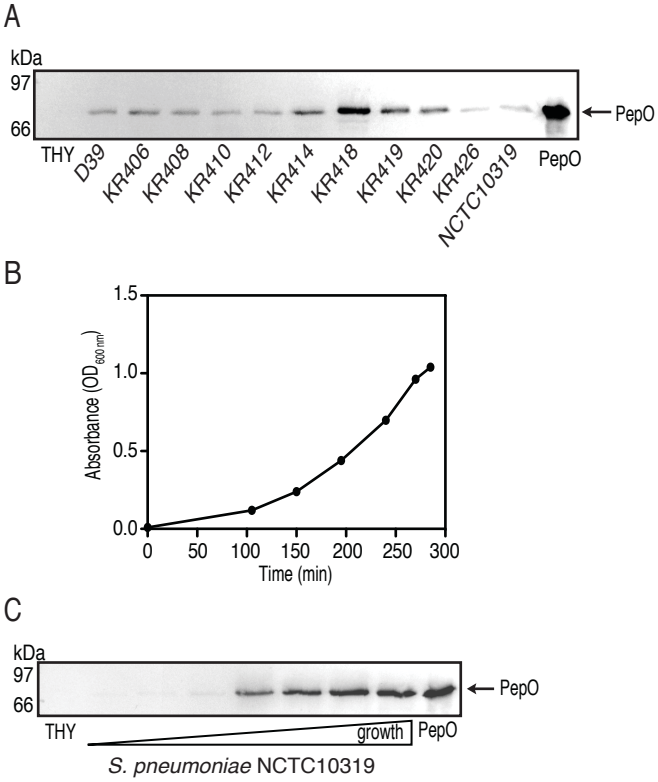

A
kDa
97
66
PepO
THY
D39
KR406
KR408
KR410
KR412
KR414
KR418
KR419
KR420
KR426
NCTC10319
PepO
B
Absorbance (OD600 nm)
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Time (min)
C
kDa
97
66
PepO
THY
growth
PepO
S. pneumoniae NCTC10319

Figure 3

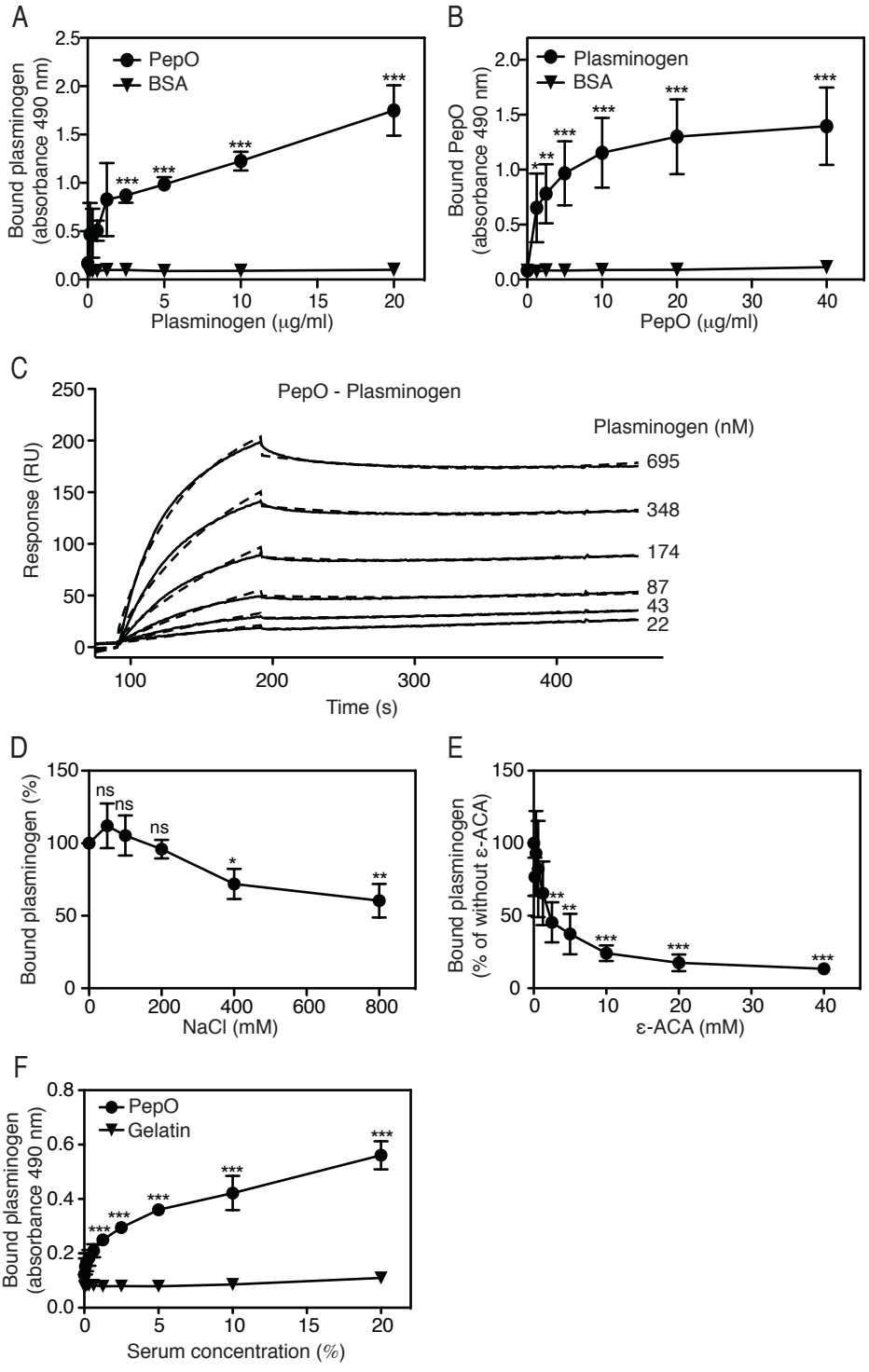

A
PepO
BSA
Bound plasminogen (absorbance 490 nm)
Plasminogen (μg/ml)
B
Plasminogen
BSA
Bound PepO (absorbance 490 nm)
PepO (μg/ml)
C
PepO - Plasminogen
Plasminogen (nM)
695
348
174
87
43
22
Response (RU)
Time (s)
D
Bound plasminogen (%)
NaCl (mM)
ns
E
Bound plasminogen (% of without ε-ACA)
ε-ACA (mM)
F
PepO
Gelatin
Bound plasminogen (absorbance 490 nm)
Serum concentration (%)

Figure 4

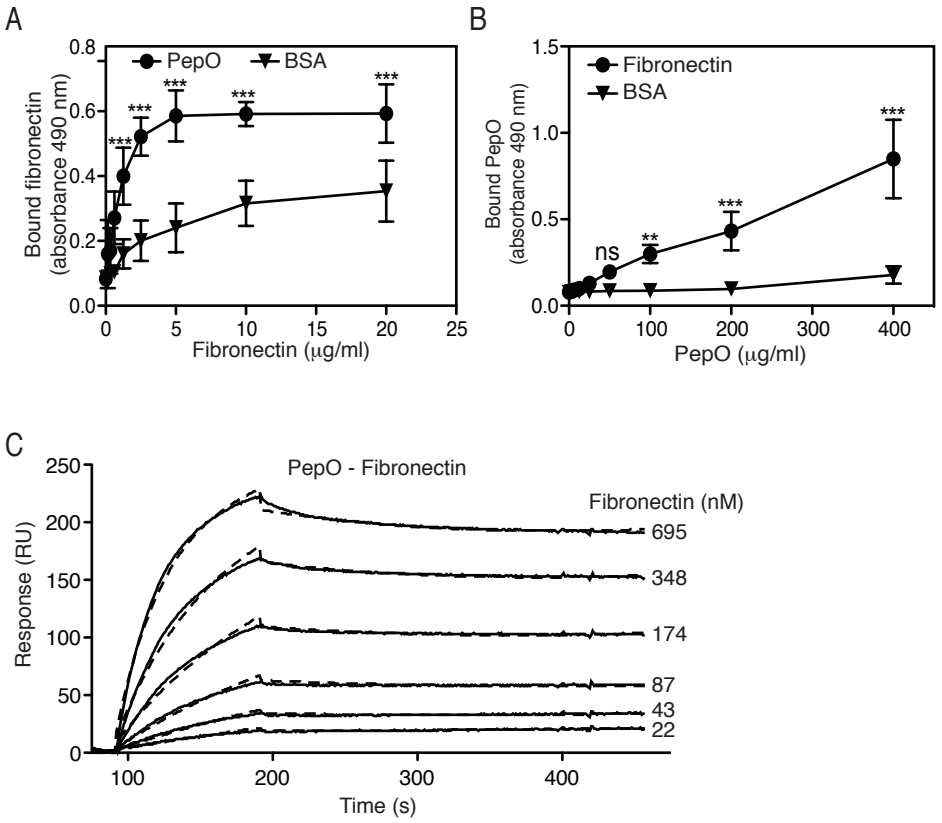
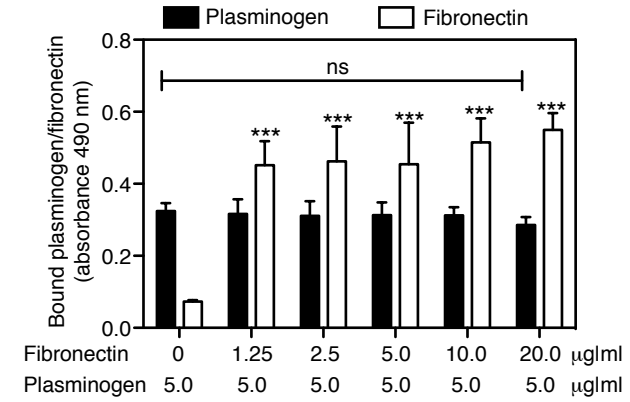

A
PepO
BSA
Bound fibronectin (absorbance 490 nm)
Fibronectin (μg/ml)
B
Fibronectin
BSA
Bound PepO (absorbance 490 nm)
PepO (μg/ml)
ns
C
PepO - Fibronectin
Fibronectin (nM)
695
348
174
87
43
22
Response (RU)
Time (s)

Figure 5

A


Plasminogen
Fibronectin
Bound plasminogen/fibronectin (absorbance 490 nm)
0.8
0.6
0.4
0.2
0.0
ns
***
Fibronectin 0 1.25 2.5 5.0 10.0 20.0 μglml
Plasminogen 5.0 5.0 5.0 5.0 5.0 5.0 μglml

B

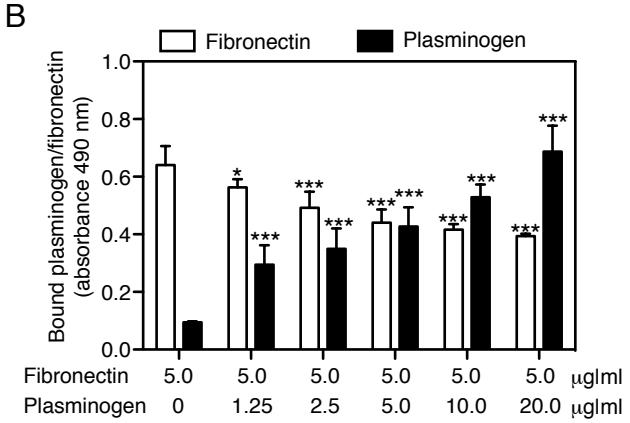

Fibronectin
Plasminogen
Bound plasminogen/fibronectin (absorbance 490 nm)
1.0
0.8
0.6
0.4
0.2
0.0
*
***
Fibronectin 5.0 5.0 5.0 5.0 5.0 5.0 μglml
Plasminogen 0 1.25 2.5 5.0 10.0 20.0 μglml

Figure 6

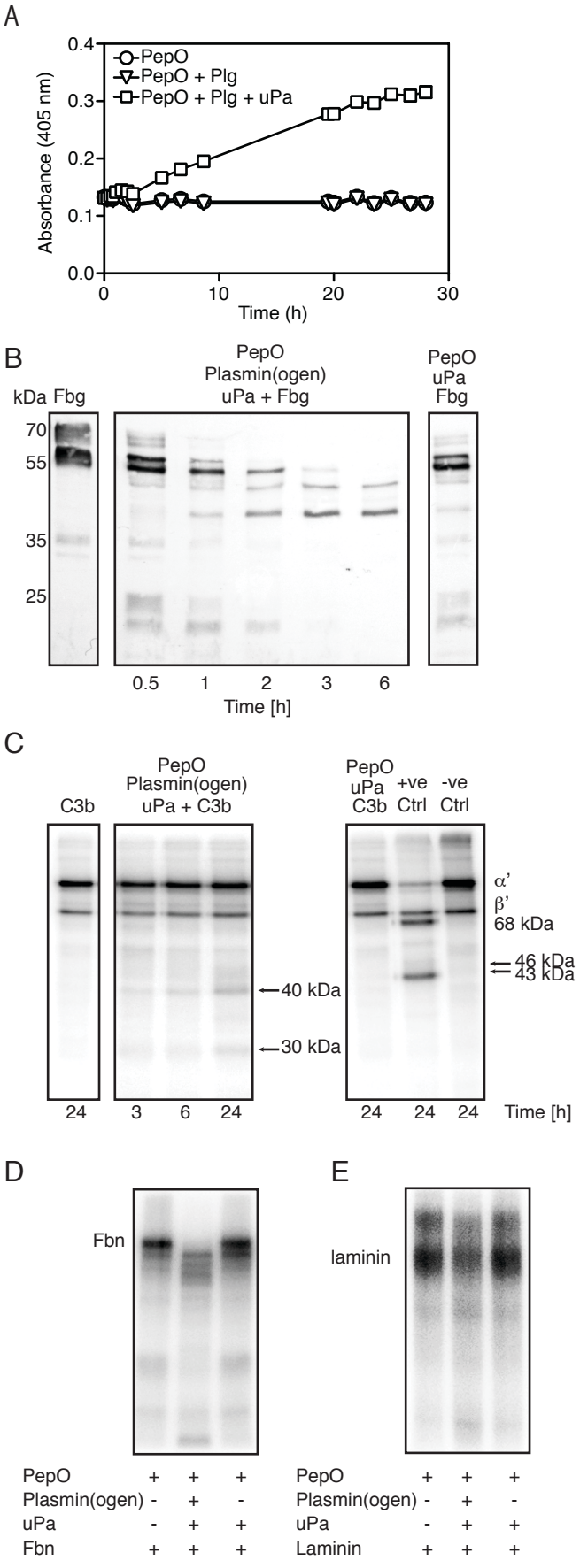
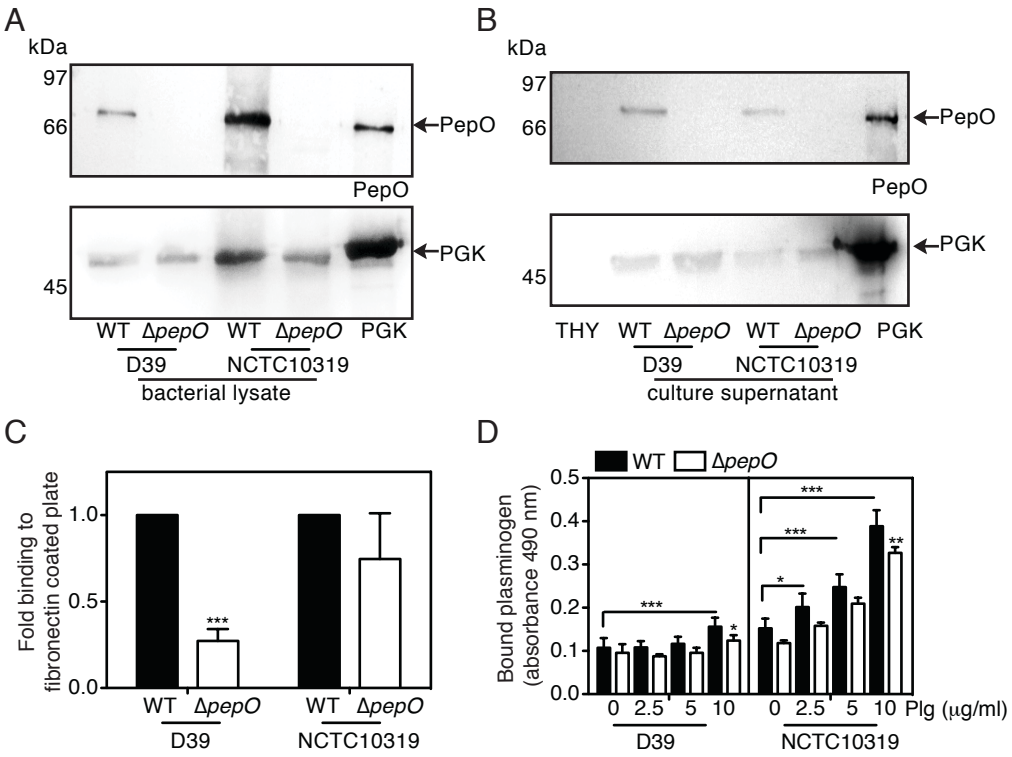

A
PepO
PepO + Plg
PepO + Plg + uPa
Absorbance (405 nm)
Time (h)
B
kDa Fbg
PepO
Plasmin(ogen)
uPa + Fbg
PepO
uPa
Fbg
70
55
35
25
0.5
1
2
3
6
Time [h]
C
C3b
PepO
Plasmin(ogen)
uPa + C3b
PepO
uPa
C3b
+ve
Ctrl
-ve
Ctrl
α'
β'
68 kDa
46 kDa
43 kDa
40 kDa
30 kDa
24
3
6
24
24
24
24
Time [h]
D
Fbn
PepO + + +
Plasmin(ogen) - + -
uPa - + +
Fbn + + +
E
laminin
PepO + + +
Plasmin(ogen) - + -
uPa - + +
Laminin + + +

Figure 7


A
kDa
97
66
PepO
PepO
45
PGK
WT ΔpepO WT ΔpepO PGK
D39
NCTC10319
bacterial lysate
B
kDa
97
66
PepO
PepO
45
PGK
THY WT ΔpepO WT ΔpepO PGK
D39
NCTC10319
culture supernatant

C
Fold binding to fibronectin coated plate
1.0
0.5
0.0
***
WT ΔpepO WT ΔpepO
D39
NCTC10319
D
WT ΔpepO
Bound plasminogen (absorbance 490 nm)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
*
**
*
0 2.5 5 10 0 2.5 5 10 Plg (μg/ml)
D39
NCTC10319

Figure 8

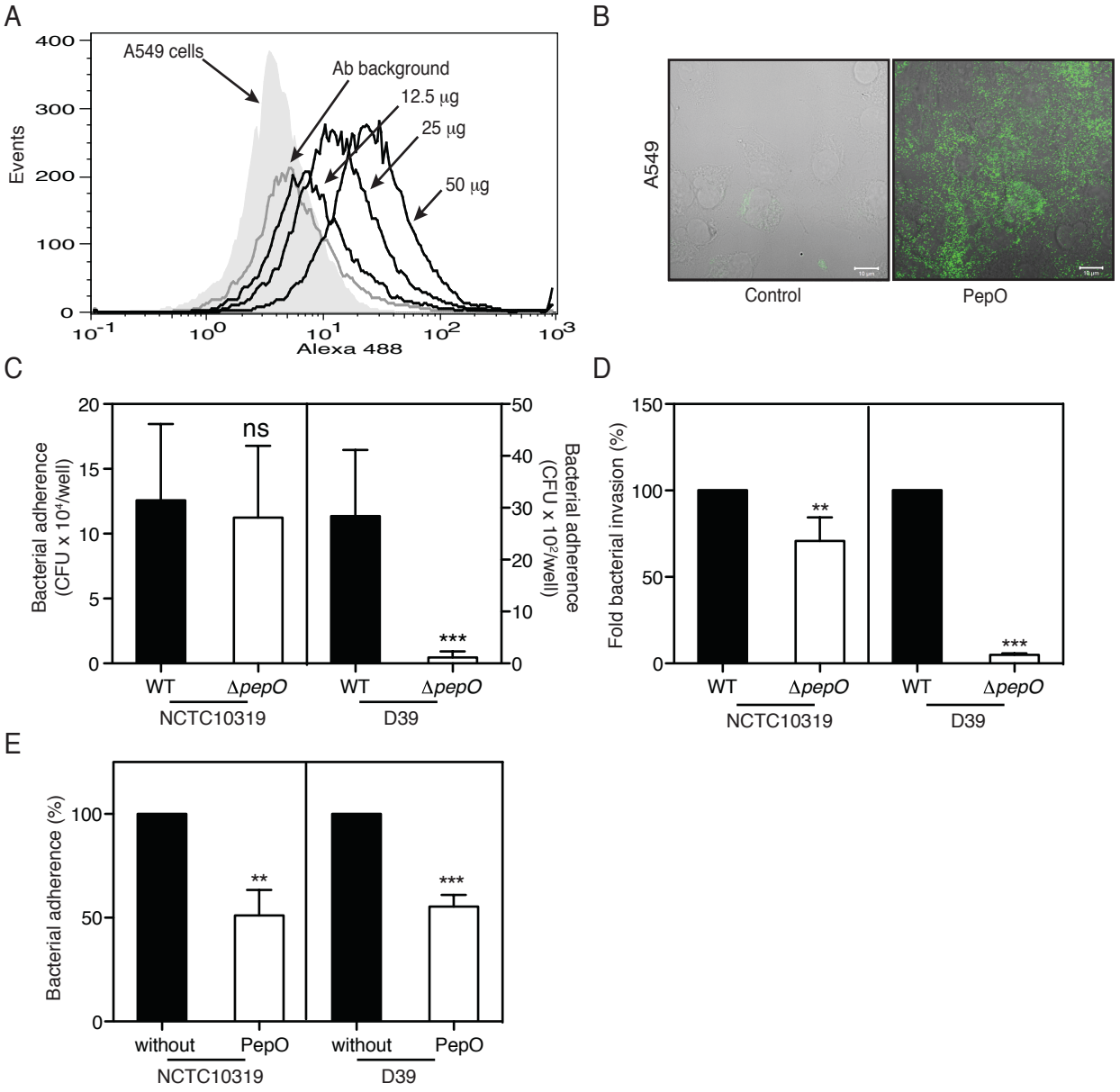
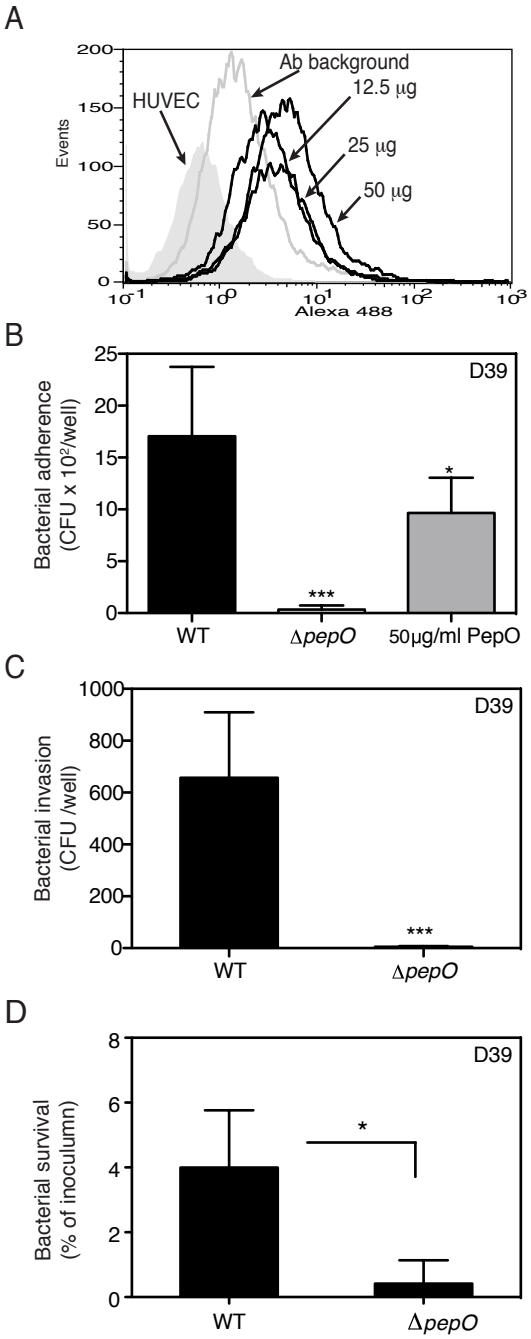

A
A549 cells
Ab background
12.5 µg
25 µg
50 µg
Events
Alexa 488
B
A549
Control
PepO
C
ns
***
Bacterial adherence (CFU x 10⁴/well)
Bacterial adherence (CFU x 10²/well)
WT
ΔpepO
NCTC10319
D39
D
**
***
Fold bacterial invasion (%)
E
**
***
Bacterial adherence (%)
without
PepO

Figure 9


A
Ab background
12.5 μg
HUVEC
25 μg
50 μg
Events
Alexa 488
B
D39
Bacterial adherence (CFU x 10²/well)
***
*
WT
ΔpepO
50μg/ml PepO
C
D39
Bacterial invasion (CFU /well)
***
WT
ΔpepO
D
D39
Bacterial survival (% of inoculumn)
*
WT
ΔpepO