Dihydrolipoamide Dehydrogenase of *Pseudomonas aeruginosa* Is a Surface-Exposed Immune Evasion Protein That Binds Three Members of the Factor H Family and Plasminogen

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Dihydrolipoamide Dehydrogenase of *Pseudomonas aeruginosa*

Is a Surface-Exposed Immune Evasion Protein That Binds Three Members of the Factor H Family and Plasminogen

Teresia Hallström,* Matthias Mörgelin,† Diana Barthel,* Marina Raguse,* Anja Kunert,* Ralf Hoffmann,‡ Christine Skerka,* and Peter F. Zipfel*–§

The opportunistic human pathogen *Pseudomonas aeruginosa* causes a wide range of diseases. To cross host innate immune barriers, *P. aeruginosa* has developed efficient strategies to escape host complement attack. In this study, we identify the 57-kDa dihydrolipoamide dehydrogenase (Lpd) as a surface-exposed protein of *P. aeruginosa* that binds the four human plasma proteins, Factor H, Factor H-like protein-1 (FHL-1), complement Factor H-related protein 1 (CFHR1), and plasminogen. Factor H contacts Lpd via short consensus repeats 7 and 18–20. Factor H, FHL-1, and plasminogen when bound to Lpd were functionally active. Factor H and FHL-1 displayed complement-regulatory activity, and bound plasminogen, when converted to the active protease plasmin, cleaved the chromogenic substrate S-2251 and the natural substrate fibrinogen. The Lpd of *P. aeruginosa* is a rather conserved gene; a total of 22 synonymous and 3 nonsynonymous mutations was identified in the lpd gene of the 5 laboratory strains and 13 clinical isolates. Lpd is surface exposed and contributes to survival of *P. aeruginosa* in human serum. Bacterial survival was reduced when Lpd was blocked on the surface prior to challenge with human serum. Similarly, bacterial survival was reduced up to 84% when the bacteria was challenged with complement active serum depleted of Factor H, FHL-1, and CFHR1, demonstrating a protective role of the attached human regulators from complement attack. In summary, Lpd is a novel surface-exposed virulence factor of *P. aeruginosa* that binds Factor H, FHL-1, CFHR1, and plasminogen, and the Lpd-attached regulators are relevant for innate immune escape and most likely contribute to tissue invasion. *The Journal of Immunology,* 2012, 189: 000–000.

*P. aeruginosa* is an opportunistic human pathogen that can reside as a harmless commensal, but can also cause severe diseases, including pneumonia and septicemia (1). *P. aeruginosa* is a major cause of hospital-acquired infections, particularly in immunocompromised individuals. In cystic fibrosis patients, this Gram-negative bacterium is a major cause of chronic lung infections (2–4). *P. aeruginosa* infections are difficult to treat, and as antibiotic-resistant strains are developing worldwide, there is a growing need to understand the crosstalk of this human pathogenic bacterium with the host immune system in detail and to develop efficient and suitable drugs to control severe *P. aeruginosa* infections (5).

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the Factor I-mediated cleavage of C3b (6–8). The complement Factor H-related protein 1 (CFHR1) is a member of the Factor H protein family, and the protein is transcribed from a separate gene. The corresponding plasma protein is composed of 5 SCRs and appears as two isoforms with either two, 42-kDa (CFHR1β), or one, 37-kDa (CFHR1α) attached carbohydrate chains (9). CFHR1 inhibits complement at level of the C5 convertases and blocks TCC assembly and formation (10).

To survive and establish an infection, any pathogenic microbe must control, inactivate, and evade the innate immune response of the host, including the complement attack. Acquisition of soluble host complement regulators is one common and important evasion strategy used by many, virtually all pathogenic microbes. P. aeruginosa, similar to other pathogenic bacteria, uses multiple strategies to actively evade and control host innate immunity and complement attack. P. aeruginosa binds the complement regulators Factor H, FHL-1, and CFHR1, and the attached regulators inhibit complement activation at the bacterial surface, block the generation of the opsonin C3b, and increase serum resistance (11). Elongation factor Tuf, a 43-kDa surface protein, was identified as the first Factor H, FHL-1, and CFHR1-binding protein of this Gram-negative pathogen (11). In addition, P. aeruginosa secretes the endogenous proteases elastase and alkaline protease, which degrade and inactivate human complement components, including C1q, C2, and C3b (12, 13). Degradation of the opsonin C3b reduces phagocytosis of the pathogen by human neutrophils, and degradation of C3 inhibits fibroblast cell growth (12, 14, 15). In addition, P. aeruginosa produces an alginate layer that forms a mechanical barrier and that inhibits complement at the level of the C5 convertases and blocks phagocytosis (16, 17).

Following interaction with the complement system, many pathogens leave the site of infection and disseminate into deeper tissue layers. For this purpose, pathogenic microbes interact with and use either pathogen-encoded or host-derived proteolytic enzymes to degrade the extracellular matrix (ECM). The 92-kDa human serum protein plasminogen is a human proenzyme that is acquired by several pathogenic microbes (18). Plasminogen is composed of five consecutive kringle domains (K1–K5) that are linked to a protease domain (P) (19). When activated by endogenous activators like tissue-type plasminogen activator or urokinase-type plasminogen activator (uPA), the protease plasmin degrades ECM components like fibrinogen, fibronectin, vitronectin, and laminin and regulates cell migration, coagulation, fibrinolysis, inflammation, wound healing, and tissue remodeling (18, 19). Plasminogen is acquired by several pathogens, including P. aeruginosa, Streptococcus pneumoniae, Borrelia burgdorferi, Staphylococcus aureus, Lactobacillus johnsonii, and Candida albicans for ECM interaction, and activated plasmin is then used for destruction of host basement membranes and ECM (11, 20–23).

In the current study, we identify P. aeruginosa dihydrodipamamide dehydrogenase (Lpd) as a novel bacterial host regulator-binding protein, and thus as a multifunctional bacterial protein. Lpd is exposed at the bacterial surface and binds the human plasma proteins Factor H, FHL-1, CFHR1, and also plasminogen. Bound to Lpd, the four human plasma proteins aid in complement evasion as well as ECM degradation. Taken together, Lpd is a novel virulence factor that mediates complement evasion and may facilitate tissue invasion of the Gram-negative bacterium P. aeruginosa. The identification of a new virulence factor and immune evasion protein of P. aeruginosa shows the complexity of the bacterial host immune crosstalk and offers new insights into the interaction of P. aeruginosa with the human host.

Materials and Methods

**Bacterial strains and culture conditions**

*P. aeruginosa* strains American Type Culture Collection (ATCC) 27853; National Collection of Type Cultures (NCTC) 10662, SG137, and PAO1; and the PAO1 derivative AH377 (24) were routinely cultured in enriched nutrient broth (NB; Serva, Amstetten, Austria) at 37°C. Thirteen clinical isolates were derived from patients with different diseases. Cultures were grown to an OD600 of ~1.0. Transformed *Escherichia coli* M15-expressing Lpd were grown in Luria Bertani liquid broth supplemented with 25 μg/ml kanamycin and 10 μg/ml carbenicillin.

**Biotinylation and isolation of *P. aeruginosa* surface proteins**

Surface biotinylation of intact *P. aeruginosa* was performed, as described (25). Briefly, bacteria (2 × 10^11) were washed in buffer A (PBS, 1 mM CaCl2, 0.5 mM MgCl2) and resuspended in buffer B (buffer A supplemented with 1.6 mM D-biotin). Bacteria were pelleted, and surface proteins were labeled with biotin by incubation with 500 μl 400 μM EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Bonn, Germany) for 30 min on ice. Cells were washed extensively in buffer C (50 mM Tris [pH 7.4], 100 mM NaCl, 27 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2) and resuspended in buffer C supplemented with protease inhibitors (Complete; Roche, Mannheim, Germany). Bacteria were lysed by sonication, and biotinylated surface proteins were purified by affinity chromatography using Immunopure Immobilized Monomeric Avidin (Pierce). Proteins were eluted with D-biotin (2 mM in PBS), according to the manufacturer’s recommendations. The elute fractions were separated by SDS-PAGE and transferred to a membrane, and biotinylated proteins were identified by HRP-conjugated avidin (Roche).

**Isolation of *F. heparinum* ligands with magnetic beads**

Factor H SCR 8–20 (40 μg) was covalently coupled to magnetic beads (100 μl suspension), according to the manufacturer’s instructions (Invitrogen, Karlsruhe, Germany). Then the beads were incubated with a preparation of purified biotinylated *P. aeruginosa* surface proteins for 2 h at 37°C. After washing three times with 50 mM HEPES (pH 7.5), 1% Nonidet P-40, 1 mM dithioerythritol, 1 mM MgCl2, and 1 mM CaCl2, proteins were eluted with 40 μl 1 M NaCl for 10 min at 37°C, separated by SDS-PAGE, and visualized by silver staining. Individual bands were excised from the gel and analyzed by mass spectrometry.

**Protein identification by peptide mass fingerprinting**

Silver-stained bands were destained using the ProteoSilver Plus silver stain kit (Sigma-Aldrich, Steinheim, Germany), and Cyano-silane-stained bands were destained, according to the manufacturer’s protocol. Subsequently, proteins were reduced and alkylated using 10 mM DTT and 100 mM iodoacetamide in 25 mM NH4HCO3, washed with 25 mM NH4HCO3, and dehydrated with acetonitrile. Digestion with trypsin, MALDI-TOF-mass spectrometry (MS), and protein identification by peptide mass fingerprinting were performed, as described (25).

**Expression and purification of recombinant proteins**

The *lpd* gene (GenBank accession no. Q91331; BankIt1555812 Seq1 JX475907; BankIt1555812 Seq2 JX475908; BankIt1555812 Seq3 JX475909; BankIt1555812 Seq4 JX475910; BankIt1555812 Seq5 JX475911; BankIt1555812 Seq6 JX475912; BankIt1555812 Seq7 JX475913; BankIt1555812 Seq8 JX475914; BankIt1555812 Seq9 JX475915; BankIt1555812 Seq10 JX475916; BankIt1555812 Seq11 JX475917; BankIt1555812 Seq12 JX475918; BankIt1555812 Seq13 JX475919; BankIt1555812 Seq14 JX475920; BankIt1555812 Seq15 JX475921; BankIt1555812 Seq16 JX475922) was amplified from genomic DNA of *P. aeruginosa* strain PA01. The amplicon was cloned into pMAL-p2X (New England Biolabs, Beverly, MA) and expressed in the *E. coli* strain M15, and protein expression was induced by 1 mM isopropyl β-d-thiogalactoside. Recombinant Lpd was sequenced by MALDI-TOF-mass spectroscopy (MS), and protein identification by peptide mass fingerprinting were performed, as described (25).

**Expression vector pQE**

The expression vector pQE (Qiagen) is a plasmid containing the *E. coli* strain M15, and protein expression was induced by 1 mM isopropyl β-d-thiogalactoside. Recombinant Lpd was sequenced by MALDI-TOF-MS and expressed in the *E. coli* strain Top10 Cloning vectors pCR4-Blunt-TOPO or pCR2.1Blunt-TOPO.
Abs
Polyconal Lpd antiserum was raised by immunizing rabbits i.m. with 200 µg purified recombinant Lpd emulsified in CFA (Difco and BD Bioscienes, Heidelberg, Germany) and boosted on days 18 and 36 with the same dose of protein in IFA. Blood was drawn 3 wk later. Generation of the human Factor H-specific mAbs, mAb E22 against SCR 3, and mAb C18 against SCR 20 has been described (28). Alexa fluor 488–conjugated polyclonal goat anti-rabbit was purchased from Molecular Probes, and the HRP-conjugated rabbit anti-goat, HRP-conjugated swine anti-rabbit, and HRP-conjugated rabbit anti-mouse were obtained from Dako (Glostrup, Denmark). A mouse His mAb was purchased from Qiagen. Polyclonal goat anti-Factor H and polyclonal goat anti-human C3 were obtained from Complement Technology (Tyler, TE), and polyclonal goat anti-plasminogen from Acris Antibodies (Herford, Germany). CFHR1 was detected with the mAb JHD10 (10) or rabbit CFHR1 antiserum (29). Fibrinogen degradation was assayed by rabbit anti-human fibrinogen (Calbiochem, Nottingham, U.K.).

ELISA
Microtiter plates (96 Maxisorb, Nunc-ImmuNoModule) were coated with Lpd (5 µg/ml) or gelatin (5 µg/ml) overnight at 4°C or with live bacteria (0.5 × 10⁶/well) for 1 h at 37°C. The plates were washed four times with PBS containing 0.1% Tween (20 PBS-Tw) and blocked for 1 h with PBS supplemented with 2% BSA (PBS-BSA) or blocking buffer I (Applichem, Darmstadt, Germany). After washing, the plates were incubated for 1 h at room temperature (RT) with Factor H (Complement Technology) (0.08–0.67 µM); FHL-1 (0.07–0.6 µM); equimolar amounts of Factor H, FHL-1, CFHR1, and plasminogen (0.13 µM); and equimolar amounts of the various Factor H constructs (0.13 µM), CFHR1 (0.07–0.58 µM), the two CFHR1 constructs (0.27 mM), plasminogen (Chromogenix, Milano, Italy) (0.001–0.54 µM), recombinant plasminogen fragments (10 µg/ml), or Lpd antiserum (1:1000). Thereafter, the wells were washed, and goat anti-human Factor H (1:500 to detect SCRs 8–11 and 1:2000 for the other deletion mutants), rabbit CFHR1 antiseraum (1:1000), goat anti-plasminogen (1:1000 to detect K1-5-P and K1-5, 1:750 to detect K4-P, and 1:500 to detect K5), or mouse anti-CFHR1 mAb (JHD10) (1:500) was added in PBS-BSA for 1 h at RT. After washing, the plates were incubated for 1 h at RT with blocking buffer I (Applichem) and incubated with plasminogen (20 µg/ml) overnight at 4°C, washed, and incubated with uPA (160 ng/ml) together with fibrinogen (10 µg/ml) at 37°C. The reaction was terminated at various time points (0.5–6 h) by addition of SDS-PAGE sample buffer (RotiLoad1; Carl Roth). Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting. Fibrinogen degradation was analyzed using a rabbit anti-human fibrinogen (1:1000) that was added for 1 h, followed by HRP-conjugated swine anti-rabbit (1:2500) added for 40 min. Development was performed with ECL Western blotting detection reagents (Applichem).

Flow cytometry
The expression of Lpd on the surface of P. aeruginosa was analyzed by flow cytometry. The P. aeruginosa strain SG137 was grown to an OD₆₀₀ ∼1.0 and washed once in PBS-BSA, and then bacteria (10⁶) were incubated with rabbit Lpd antiserum (1:100) for 45 min at 4°C. Bacteria were washed and incubated for 30 min with Alexa fluor 488-conjugated goat anti-rabbit (1:2000), washed, and analyzed by flow cytometry (FACScan LRII; BD Biosciences, Mountain View, CA). All incubations were kept in PBS-BSA, and secondary pAb was added separately as a negative control.

Transmission electron microscopy
The expression of Lpd on the surface of P. aeruginosa and the binding of Factor H and CFHR1 were analyzed by negative staining and electron microscopy, as described (31). Rabbit Lpd antiserum, plasma-purified human Factor H, or recombinant CFHR1 was labeled with 5 nm colloidal gold, 15 nm colloidal gold, or 45 nm colloidal gold, respectively. Bacteria (SG137) were mixed with the Abs or Factor H and/or CFHR1, and 5 µl aliquots were added onto carbon-coated grids for 1 min, washed with two drops of water, and stained on two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were observed in a Joed JEM 1230 electron microscope (JEOL, Tokyo, Japan) operated at 60 kV accelerating voltage. Images were recorded with a Multiscan 791 CCD camera (Gatan, Pleasanton, CA).

Isolation of P. aeruginosa outer membrane proteins
Bacteria grown to exponential phase were washed with 50 mM Tris-HCl (pH 8.0). The pellet was resuspended in 50 mM Tris–HCl (pH 8.0) containing 3% Empigen (Calbiochem) and protease inhibitors (Complete; Roche). Outer membrane proteins (OMPs) were extracted by rotating the mixture at 37°C for 2 h. The bacterial cells, stripped of their outer membranes, were centrifuged, and the supernatants were collected. Thereafter, the supernatants were subjected to SDS-PAGE, transferred to a membrane, and analyzed by Western blotting using a rabbit Lpd antiserum (1:500) for 1 h, followed by HRP-conjugated swine anti-rabbit (1:2500) for 40 min. Development was performed with ECL Western blotting detection reagents (Applichem).

Sequencing of the lpd gene
The lpd gene of the 5 P. aeruginosa strains (SG137, ATCC 27853, NCTC 10662, PA01, AH377) and of 13 clinical isolates was amplified by PCR

<table>
<thead>
<tr>
<th>Plasminogen Fragment</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1-K5-P</td>
<td>Forward</td>
<td>GGTACCACGAGCCCTCGGATGAC</td>
</tr>
<tr>
<td>K1-K5-P</td>
<td>Reverse</td>
<td>GGCCGAGCCATTTTCTCATACCT</td>
</tr>
<tr>
<td>K4-K5-P</td>
<td>Forward</td>
<td>GGTACCTGACACACCTGGACAGC</td>
</tr>
<tr>
<td>K4-K5-P</td>
<td>Reverse</td>
<td>GGCCGGCCATTTTCTCATACCT</td>
</tr>
<tr>
<td>K5-P</td>
<td>Forward</td>
<td>GGTACCTGACACTTGATGGTGGG</td>
</tr>
<tr>
<td>K5-P</td>
<td>Reverse</td>
<td>GGCCGGCCATTTTCTCATACCT</td>
</tr>
<tr>
<td>K1-K5</td>
<td>Forward</td>
<td>GGTACCTGAGGTCTACTCAGG</td>
</tr>
<tr>
<td>K1-K5</td>
<td>Reverse</td>
<td>TCTAGAGCTAGCTAGGGACATC</td>
</tr>
</tbody>
</table>
using HotStarTaq (Qiagen) using the primers 5'-TAGCGTTTCTCTTCA GCCGC-3' and 5'-CCGGTGCGCTTGTGACGC-3'. The sequence of the amplified products was determined using an ABI 3100 sequencer (Applied Biosystems). The sequences were compared with that of the lpd gene of P. aeruginosa PA01 (GenBank accession no. Q913D1).

Serum bactericidal assay

The five P. aeruginosa strains (SG137, ATCC 27853, NCTC 10662, PA01, AH377) were grown to an OD$_{600}$ of 1.0 and diluted in Mg-EGTA buffer. Bacteria (10$^6$ CFU) were incubated in normal human serum (NHS), used at increasing concentrations (0–50%), NHS or complement active HS (ATCC 27853, 20%; NCTC 10662, 15%; PA01, 50%; AH377, 50%), or depleted serum reconstituted with purified Factor H (100 µg/mL) in a final volume of 100 µL at 37°C. After 0 and 60 min, 10 µL aliquots were removed and spread onto NB plates. After 18 h of incubation at 37°C, CFU were determined. In the inhibition experiments, bacteria (strain SG137) were preincubated with Lpd antisera (1:20). Factor H deletion mutants SCRs 18–20 (25 µg/mL), or SCRs 8–11 (25 µg/mL) for 10 min at RT. After washing, bacteria were challenged with 10% NHS in a final volume of 100 µL at 37°C. In addition, bacteria were incubated with an Efb antiserum (1:20), directed against the S. aureus protein Efb, which is absent on the surface of P. aeruginosa, or 5 µL rabbit IgGs.

Statistics

Results were analyzed by Student t test for paired data. The p value of $p < 0.05$ was considered to be statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

Identification of a second Factor H-binding protein of P. aeruginosa

To identify additional Factor H-binding proteins of P. aeruginosa, surface proteins of P. aeruginosa strain PA01 were biotinylated, extracted, and absorbed to a Factor H/SCR 8–20 matrix. Bound proteins were eluted, separated by SDS-PAGE, and visualized by silver staining. This approach identified two major bands with mobilities of $\sim 57$ and 43 kDa (data not shown). The 57-kDa band was identified as Lpd, and the 43 kDa band as Tuf, the known flavoprotein with a predicted molecular mass of 50.2 kDa (32).

Recombinant expression of Lpd and generation of polyclonal Lpd antiserum

P. aeruginosa lpd gene was amplified, the corresponding PCR product was cloned into expression vector pQE, recombinantly expressed in E. coli as a N-terminal His-tagged protein, and purified by nickel chelate chromatography. The bacterial lysate, flow-through (FT), wash (w), and elute (e) fractions were separated by SDS-PAGE, and proteins were visualized by silver staining. This approach identified in the elute fraction two bands, one of 57 and one of 50 kDa (Fig. 1A, lane 4). In addition, a His antisera identified the 57-, but not the 50-kDa band (Fig. 1B, lane 3). Purified recombinant Lpd was used to immunize rabbits, and the corresponding polyclonal Lpd antisera identified both the 57- and 50-kDa bands (Fig. 1B, lane 3). The two bands were excised from the gel and analyzed by mass spectrometry, and both the upper and the lower band were identified as Lpd. The reactivity with the His- and the Lpd-specific antisera, together with the mass spectrometry data, suggests that the 57-kDa band represents the full-length His-tagged Lpd protein, and the 50-kDa band most likely is a degradation product that has the His tag cleaved off.

Recombinant Lpd binds Factor H and FHL-1

To confirm that Lpd of P. aeruginosa is a Factor H-binding protein, binding of Factor H to Lpd was assayed. Factor H bound to immobilized Lpd, and binding was dose dependent (Fig. 2B). Factor H contains two heparin binding domains located in SCR 7 and SCRs 19–20, and FHL-1 contains one heparin binding domain in SCR 7 (33). Therefore, we asked whether heparin affects the Lpd/Factor H or Lpd/FHL-1 interaction. Heparin inhibited binding of both human regulators, and the effect was dose dependent (Fig. 2C, 2D). Heparin used at 1 mg/ml inhibited Factor H binding to Lpd by 73% and FHL-1 binding by 66%.

To localize the region(s) of Factor H and FHL-1 that contacts Lpd, a series of Factor H deletion mutants were tested for binding to immobilized Lpd. The N-terminal SCRs 1–7 (FHL-1) and the C-terminal constructs SCRs 15–20 and SCRs 18–20 bound to Lpd (Fig. 2E). Deletion fragments SCRs 1–4, SCRs 1–5, SCRs 1–6, SCRs 8–11, SCRs 11–15, SCRs 15–18, and SCRs 19–20 bound either with reduced intensity or did not bind. Thus, the human complement regulator Factor H binds to Lpd via two interaction domains that are included within SCR 7 and SCRs 18–20, and FHL-1 binds via one domain, that is, SCR 7. To confirm that the C terminus of Factor H binds to bacterial Lpd, epitope-specific mAbs were used as blocking agents. A mAb that binds to SCR3 (E22) and a mAb that binds to SCR 20 (C18) of Factor H were used. The mAb C18, which binds to the C terminus, blocked Factor H binding to immobilized Lpd by $>60%$ (Fig. 2F). In contrast, mAb E22, which reacts with the N terminus, did not affect Factor H binding to Lpd. Thus, this demonstrates that inhibition is not caused by steric hindrance and that the surface recognition region of Factor H (SCRs 18–20) is important for binding of Lpd.

Factor H and FHL-1 control alternative pathway-mediated complement activation at the level of the C3 convertase by acting as cofactors for the serine protease Factor I. To demonstrate that the regulators bound to Lpd are functionally active, we assayed cofactor activity of Lpd-bound Factor H or FHL-1 for C3b degradation. C3b degradation was revealed as the appearance of the degradation fragments $\alpha 43$ and $\alpha 41$ kDa. Bound to Lpd, Factor H and FHL-1 were functionally active, acting as cofactors in the degradation of C3b (Fig. 3A, 3B, lanes 3). Thus, Factor H and FHL-1 bound to P. aeruginosa Lpd maintain their central regulatory functions and control complement activation at the level of the C3 convertase.
The C-terminal surface attachment region SCRs 18–20 of Factor H forms one binding site for Lpd. Given the extensive sequence similarity of the C termini of Factor H (SCRs 18–20) and CFHR1 (SCRs 3–5) (100, 100, and 97%) (10), we asked whether CFHR1 also binds to Lpd. CFHR1 bound to immobilized Lpd, and binding was dose dependent (Fig. 4A). CFHR1 is a heparin-binding protein, and, therefore, we also assayed the effect of heparin for the CFHR1/Lpd interaction (10). Heparin affects CFHR1 binding to Lpd, and at 5 mg/ml heparin inhibited the interaction by 56% (Fig. 4B). Thus, heparin influences the binding of the complement regulator CFHR1 to P. aeruginosa Lpd.

In addition, the interacting domains of CFHR1 that are relevant for contact of Lpd were localized by assaying binding of equimolar amounts of CFHR1 deletion mutants. Both deletion mutants bound to immobilized Lpd, but CFHR1/SCRs 3–5 bound with ∼4 times higher intensity to Lpd compared with CFHR1/SCRs 1–2 (Fig. 4C). Thus, the C-terminal CFHR1/SCRs 3–5 represent the major binding region of Lpd.
**Lpd binds human plasminogen**

Several microbial Factor H-binding proteins also bind plasminogen (34–37). We therefore asked whether *P. aeruginosa* Lpd also binds plasminogen. Plasminogen bound to Lpd, and binding was dose dependent and saturated at 0.27 μM plasminogen (Fig. 5A). As lysine residues are often relevant for plasminogen binding, we assayed whether the lysine analog εACA influences the plasminogen/Lpd interaction. εACA used at 1.0 mM inhibited plasminogen binding to immobilized Lpd by 50%, and at 10 mM εACA blocked the interaction completely (Fig. 5B). Thus, lysine residues are relevant for the Lpd/plasminogen contact.

To localize the domain(s) of plasminogen that contacts Lpd, recombinant plasminogen and a series of recombinant plasminogen deletion mutants were generated, and these proteins were tested for Lpd binding. Recombinant full-length plasminogen (K1-5-P), the deletion mutant that has kringle domains 4 and 5 linked to the protease domains (i.e., K4-K5-P) and a fragment that includes all five kringle domains, but lacks the protease domain (K1-5), bound to immobilized Lpd (Fig. 5C). The fragment that includes kringle 5 and the protease domain bound to immobilized Lpd with a reduced intensity. Thus, this indicates that kringle domain 4 of plasminogen contains the major Lpd binding site.

**Lpd-bound plasminogen is accessible for uPA and is proteolytically active**

Plasminogen is a zymogen that can be converted to active plasmin (19). To determine whether Lpd-bound plasminogen is accessible for...
and converted by the physiological activator uPA to plasmin, plasminogen bound to Lpd was treated with uPA, and the activity of plasmin was assayed. Lpd-bound plasminogen was converted to plasmin, which cleaved the chromogenic substrate S-2251 (Fig. 5D).

In addition, we tested whether plasmin bound to Lpd also cleaved the physiological substrate fibrinogen. Fibrinogen was degraded by newly formed plasmin, as revealed by the decrease in intensity of the two fibrinogen bands (64 and 58 kDa). Already after 30 min fibrinogen was degraded (Fig. 5E, lane 3, upper panel), and, after 1 h, fibrinogen was no longer detectable (Fig. 5E, lane 5, upper panel). Thus, Lpd-bound plasminogen is accessible for the activator uPA and is activated to plasmin, which cleaves both a synthetic and a natural substrate.

**Competition of CFHR1 and Factor H for binding to Lpd**

Both CFHR1 and Factor H bind Lpd via their conserved C terminus. We therefore asked whether the two proteins bind simultaneously to different sites of Lpd or whether they compete for binding. CFHR1 when used at increasing concentrations slightly reduced Factor H binding to Lpd at a molar ratio of 0.1:1–1:1 and was further reduced by 53% at a ratio of 4:1 (Fig. 6A). Similarly, in a reverse setting, Factor H slightly reduced CFHR1 binding to Lpd. At a 4-fold excess of Factor H, CFHR1 binding was reduced by 50% (Fig. 6B). The latter ratios exceed the physiological level of the two regulators in human plasma, where the molar ratio is 0.35:1 (CFHR1:Factor H). These results show that CFHR1 and Factor H bind simultaneously to bacterial Lpd at physiological plasma concentrations.

**Factor H and plasminogen bind simultaneously to Lpd**

As plasminogen and Factor H also bind to Lpd, we asked whether the two proteins bind simultaneously to Lpd and whether the two proteins compete for binding. Plasminogen bound dose dependently to immobilized Lpd and did not affect Factor H binding (Fig. 6C). Similarly, Factor H when used at increasing concentrations bound dose dependently to Lpd, and plasminogen binding was not affected (Fig. 6D). Thus, the two human plasma proteins plasminogen and Factor H bind independently and most likely to different regions of the Lpd protein. At physiological plasma levels (plasminogen:Factor H molar ratio of 1:1.5, indicated by the arrow), both Factor H and plasminogen bound to Lpd.

**Lpd binds equimolar amounts of Factor H, FHL-1, CFHR1, and plasminogen**

The four human regulators, Factor H, FHL-1, CFHR1, and plasminogen, bind to Lpd. To compare the intensity of binding, each human plasma protein was bound at equimolar amounts to immobilized Lpd. Factor H, FHL-1, and CFHR1 bound to Lpd with comparable intensity and plasminogen with a stronger intensity (Fig. 6D). As Factor H, FHL-1, and CFHR1 are detected with the same antiserum, it is concluded that the three human complement proteins bind with similar intensity to Lpd. In this setup, the intensity of plasminogen binding cannot be directly compared with the other plasma proteins as plasminogen was detected with a different antiserum.

**Lpd is a bacterial surface protein**

Surface biotinylation and also binding of human regulators suggested surface expression of *P. aeruginosa* Lpd. Therefore, we analyzed Lpd expression at the bacterial surface. First, Lpd was detected at the bacterial surface by flow cytometry (Fig. 7A). In addition, when Lpd was analyzed on the surface of intact bacteria by electron microscopy, the protein was evenly distributed over the surface (Fig. 7B). Next, expression levels of Lpd were compared for the...
five P. aeruginosa strains using a whole-cell ELISA. The Lpd levels were highest on the surface of strain SG137. Lpd levels for the strains ATCC 27853, NCTC 10662, PAO1, and AH377 ranged from 50 to 80% in comparison with strain SG137 (Fig. 7C). In addition, the presence of Lpd was analyzed in the OMP fractions of the five strains. Lpd was detected in the OMP preparations of all five strains (i.e., SG137, ATCC 27853, NCTC 10662, PAO1, and AH377) as a 57-kDa band (Fig. 7D, lanes 1–5). Thus, four different and independent methods, that is, flow cytometry, electron microscopy, whole-cell ELISA, and OMP preparations, identified Lpd as a surface protein of P. aeruginosa.

P. aeruginosa has at least two Factor H, FHL-1– and CFHR1-binding proteins. Therefore, we wanted to demonstrate the role of Lpd for serum resistance. To this end, we blocked Lpd on the surface of strain SG137, which had the highest Lpd surface level using the specific Lpd antiserum. Then bacteria were challenged with NHS, and, after incubation, bacterial survival was assayed. Blockade of Lpd reduced the bacterial survival by 56% compared with the survival of SG137 in NHS (Fig. 7E). The effect was specific, as neither an unrelated antiserum nor purified rabbit IgGs affected bacterial survival. Thus, surface-exposed Lpd contributes to serum resistance of P. aeruginosa.

**Lpd sequence variation in clinical P. aeruginosa isolates**

To assay whether the sequence of the lpd gene in the different isolates is conserved or polymorphic, the lpd gene of all 13 clinical *P. aeruginosa* isolates and of the 5 laboratory strains was amplified and the sequence was determined. Sequence analysis revealed a total of 25 nucleotide exchanges among the 18 strains. Twenty-two nucleotide exchanges represented synonymous exchanges (Fig. 8A), and three nucleotide exchanges represented nonsynonymous exchanges. The nonsynonymous exchange, which causes an exchange of Asn97 to Ser, was identified in the laboratory strain ATCC 27853 and in two clinical isolates (i.e., 9 and 11) (Fig. 8C). An Ala179 to Val exchange was identified in the laboratory strain SG137, and a His306 to Tyr in the clinical isolate 1 (Fig. 8C). Thus, the lpd gene of *P. aeruginosa* is rather conserved.

To assay whether the clinical isolates of *P. aeruginosa* express Lpd in the outer membrane, the 13 clinical *Pseudomonas* isolates were tested for the presence of Lpd in the OMP fractions. Lpd was detected in the OMP fraction of all 13 *P. aeruginosa* clinical isolates as a 57-kDa band (Fig. 8B). The secondary Ab alone did not cross-react with Lpd (Supplemental Fig. 1). However, when assaying Lpd levels by whole-cell ELISA, 11 clinical isolates expressed Lpd; however, the level of expression varied (Fig. 8C). Lpd expression was highest for isolate 1, followed by the clinical isolates 2, 5, and 4. Compared with isolate 1, Lpd levels among the 10 other clinical isolates ranged from 38 to 75%. Clinical isolates 10 and 13 did not express Lpd when analyzed by whole-cell ELISA. Thus, Lpd is expressed in the outer membrane of all tested *P. aeruginosa* strains, but surface expression varies between strains.

**Distribution of Factor H and CFHR1 on the bacterial surface**

Binding and surface distribution of the two human complement regulators Factor H and CFHR1 were evaluated by electron microscopy. Used as single proteins, Factor H (Fig. 9AI) and also CFHR1 (Fig. 9AII) bound to the surface of *P. aeruginosa*, and apparently bind to the same bacterial proteins. Both proteins were distributed over the whole bacterial surface. When added together, the two proteins, labeled with either gold particles of 15 nm (Factor H) or 45 nm (CFHR1) bound in close vicinity to each other (Fig. 9AIII, black arrows), but also to distinct sites (Fig. 9AIII, white arrows). Thus, the two human proteins colocalize at the surface of *P. aeruginosa* and apparently bind to the same bacterial proteins.

**Bound to the surface of P. aeruginosa, the complement regulators Factor H, FHL-1, and CFHR1 protect against the complement attack**

We were interested to determine the contribution of all three human regulators for serum resistance. To assay whether the five laboratory *P. aeruginosa* strains varied in serum resistance and to determine the optimal serum concentration for bacterial survival in complement regulator-depleted serum, the five laboratory *P. aeruginosa*
strains were challenged with increasing concentrations of NHS. The *P. aeruginosa* strains showed differences in serum resistance (Fig. 9B). Survival of the strains SG137, ATCC 27853, and NCTC 10662 was reduced dose dependently when the NHS concentration was increased. In contrast, PAO1 and AH377 survived in all tested NHS concentrations. Therefore, the various *P. aeruginosa* strains were cultivated in different concentrations of regulator-depleted human serum to obtain optimal results. To this end, all five laboratory strains were challenged with complement active human serum in which all three regulators were depleted, that is, HS^D^FH/FHL-1/CFHR1 (Supplemental Fig. 2). After incubation, bacteria were re-
covered and survival was evaluated. In this case, survival of strain SG137 was reduced by 84% (Fig. 9C). Survival of the other strains was also reduced, but to a different extent. Survival of strain AH377 was reduced by 70%, strain ATCC 27853 by 62%, strain PAO1 by 37%, and strain NCTC 10662 by 34% (Fig. 9C). Addition of Factor H to the depleted serum reversed the effect, and survival of all three strains was increased (Fig. 9D). To confirm the protective effect of Factor H for the survival of *P. aeruginosa*, one of the Lpd binding sites within Factor H was blocked by preincubating strain SG137 with the C-terminal surface binding region SCRs 18–20 of Factor H. Then bacteria were challenged with NHS, and, after incubation, bacterial survival was assayed. Blockade of Factor H binding to *P. aeruginosa* by the C-terminal SCRs 18–20 reduced the bacterial survival by 62% (Fig. 9E). The effect was specific, as SCRs 8–11, which do not bind to *P. aeruginosa*, did not affect the bacterial survival. Thus, this demonstrates that the human complement regulators when bound to the bacterial surface protect the bacterium from the damaging effects of the activated complement system.

**Discussion**

In this study, we identify Lpd as a novel surface-exposed complement regulator-binding protein of the Gram-negative bacterium *P. aeruginosa*. Lpd binds Factor H and three additional human plasma proteins, that is, FHL-1, CFHR1, and plasminogen. The surface-bound complement inhibitors contribute to serum resistance, and bacterial survival was reduced when *P. aeruginosa* strain SG137 was challenged with complement active human serum depleted of Factor H/FHL-1/CFHR1. Lpd is a bacterial surface protein involved in serum resistance of *P. aeruginosa*. Thus, Lpd is a new virulence factor of *P. aeruginosa* that binds Factor H, FHL-1, CFHR1, and plasminogen.

Lpd is the second Factor H, FHL-1, CFHR1, and plasminogen-binding immune evasion protein identified from *P. aeruginosa*. Lpd, which has a predicted mass of 51.6 kDa, was initially identified as a cytoplasmic protein and as a component of the enzymatic pyruvate dehydrogenase complex, which catalyzes the electron transfer between pyridine nucleotides and disulfide components (32, 38, 39). Sequence analysis of the 5 laboratory strains and 13 clinical strains were challenged with increasing concentrations of NHS. The *P. aeruginosa* strains showed differences in serum resistance (Fig. 9B). Survival of the strains SG137, ATCC 27853, and NCTC 10662 was reduced dose dependently when the NHS concentration was increased. In contrast, PAO1 and AH377 survived in all tested NHS concentrations. Therefore, the various *P. aeruginosa* strains were cultivated in different concentrations of regulator-depleted human serum to obtain optimal results. To this end, all five laboratory strains were challenged with complement active human serum in which all three regulators were depleted, that is, HS^D^FH/FHL-1/CFHR1 (Supplemental Fig. 2). After incubation, bacteria were re-
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were bound to particles of 15 nm, and CFHR1 with particles of 45 nm. The two proteins were bound to *P. aeruginosa* strain SG137 either as individual proteins or in combination, and binding was determined by transmission electron microscopy. Factor H and CFHR1 colocalize at the surface of

**FIGURE 9.** Factor H and CFHR1 colocalize at the surface of *P. aeruginosa*, and the attached complement regulators protect the bacteria from complement damage. (A) Factor H and CFHR1 surface staining was evaluated by electron microscopy. Factor H was directly labeled with gold particles of 15 nm, and CFHR1 with particles of 45 nm. The two proteins were bound to *P. aeruginosa* strain SG137 either as individual proteins or in combination, and binding was determined by transmission electron microscopy. Factor H (I) and CFHR1 (II) bind to the surface of *P. aeruginosa* and are distributed over the surface. When used together (III), both proteins bind to the same spots (black arrows) and also to unique likely independent sites (white arrows). Data shown are representative of three independent experiments. (B) Serum resistance of the five laboratory *P. aeruginosa* strains was analyzed by challenging intact bacteria with NHS, used at increasing concentrations (0–50%) diluted in Mg-EGTA buffer. After incubation, the cells were plated on NB agar plates, and the number (CFU) of surviving bacteria was determined. (C) The relevance of surface-bound Factor H, FH/FHL-1, and CFHR1 was analyzed by challenging intact bacteria with complement active human serum in which the three human regulators were depleted. *P. aeruginosa* strains were incubated in HSΔFH/FHL-1/CFHR1 diluted in Mg-EGTA buffer. After incubation, the cells were plated on NB agar plates, and the number (CFU) of surviving bacteria was determined. To allow a direct comparison of the survival rates of all five bacterial strains, the fraction of bacteria obtained after cultivation in NHS was set to 100%. (D) *P. aeruginosa* strains SG137, ATCC 27853, and NCTC 10662 were incubated with NHS, HSΔFH/FHL-1/CFHR1, or HSΔFH/FHL-1/CFHR1 supplemented with purified Factor H (100 μg/ml). After incubation, the cells were plated on NB agar plates, and the number (CFU) of surviving bacteria was determined. Survival after 1 h is shown. (E) Preincubation of SCRs 18–20 of Factor H with SG137 reduced bacterial survival in NHS. Bacteria were incubated with Factor H depletion mutants SCRs 8–11 or SCRs 18–20 and were thereafter challenged with NHS (10%). After incubation, bacteria were plated on NB agar plates, and the number (CFU) of surviving bacteria was determined. The mean values of three independent experiments and SD are presented. Statistical significance of differences was estimated using Student’s t test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
S. pyogenes (30, 49, 50). In this study, we show that Lpd binds human CFHR1 and the major binding region is located within the C-terminal surface binding region SCRs 3–5. This region shows high level of sequence identity with SCRs 18–20 of Factor H (100, 101, 102) (10), thus explaining cooperative binding of the two human regulators to Lpd.

Lpd is a multifunctional bacterial protein that, in addition to Factor H, FHL-1, and CFHR1, also binds plasminogen. Recruitment of plasminogen is used by many pathogens and is a strategy for tissue penetration. Several microbial proteins bind Factor H, FHL-1, and also plasminogen. These include Tuf from P. aeruginosa, CRASPs from B. burgdorferi, BpCA from Borrelia parkeri, and M protein from S. pyogenes (11, 22, 36, 51, 52). Plasminogen is composed of five kringle domains each of ~80 aa in size linked to a protease domain. This human plasma protein also binds fibrinogen and to other ECM proteins. Apparently, plasminogen is recruited by microorganisms via different domains. Lpd attaches plasminogen to fibrinogen and other ECM proteins. Fibrinogen is cleaved by active protease plasmin, cleaved the synthetic chromogenic substrate. This degradation allows ECM components such as fibrinogen, laminin, fibronectin, and fibronectin. This results in the release of intact bacteria. This binding profile suggests that, in addition to Factor H, Lpd, as well as other human regulators to Lpd, uses additional bacterial complement regulator-binding proteins to bind Factor H and CFHR1. At present, the two Pseudomonas proteins Lpd and Tuf have been identified to bind Factor H, FHL-1, and CFHR1 (11). The relevance of surface-attached human complement regulators was determined by challenging intact bacteria with complement active, Factor H, FHL-1, and CFHR1-depleted human serum, and then survival of bacteria was evaluated. In this case, bacterial survival was reduced by up to 84%. Addition of Factor H to the depleted serum resulted in an increase in survival of P. aeruginosa. Thus, Factor H when attached to the surface protects the pathogenic bacterium from complement damage and contributes to serum resistance. The damaging effect of the depleted human serum was strain dependent and ranged from 84 to 34%. However, the survival rate did not directly correlate with Lpd surface levels. This suggests that P. aeruginosa uses additional bacterial complement regulator-binding proteins. Thus, Lpd of P. aeruginosa is a novel bacterial virulence factor expressed at the bacterial surface, which acquires human complement regulators and consequently protects the bacterium from complement attack. The elucidation of the mechanisms that P. aeruginosa uses to interact with the human host and identification of a novel P. aeruginosa virulence and immune evasion protein identify novel candidates for vaccine development.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary Fig. 1: Lpd is present in the outer membrane protein (OMP) preparation of all 13 clinical *P. aeruginosa* isolates. Lpd is detected in the outer membrane fractions of all tested clinical isolates as a single band of 57 kDa. A fraction containing outer membrane proteins was prepared from the thirteen clinical *P. aeruginosa* isolates, separated by SDS-PAGE, transferred to a membrane and analyzed by Western blotting using the Lpd antiserum. The secondary antibody used alone did not react with Lpd or any other proteins in the OMP preparation (antibody control, bottom panel).

**Preparation of OMPs of *P. aeruginosa***

*P. aeruginosa* clinical isolates were grown to exponential phase, then bacteria were washed in 50 mM Tris-HCl (pH 8.0) and pelleted by centrifugation. The bacterial pellet was resuspended in 50 mM Tris-HCl (pH 8.0) containing 3 % Empigen (Calbiochem) and protease inhibitors (Complete, Roche). OMPs were extracted by rotating the mixture at 37°C for 2 h. Thereafter, the bacterial cells, stripped of their outer membranes, were centrifuged and the OMP containing supernatants were subjected to SDS-PAGE, transferred to a membrane and Lpd expression was analyzed by Western blotting using a rabbit Lpd antiserum (1:500) and HRP-conjugated swine anti-rabbit (1:2500). The membrane was developed with ECL Western blotting detection reagents (Applichem). The secondary antibody alone was used as a negative control.

*Lpd is present in the OMP fraction*
Lpd was identified in the OMP fraction of all thirteen *P. aeruginosa* clinical isolates as a single band of 57 kDa (Fig. 1, *upper panel*). The secondary antibody did not cross react with Lpd (Fig. 1, *lower panel*).
Supplementary Fig. 2: Human serum depleted of Factor H, FHL-1 and CFHR1 is complement active. A, Alternative pathway mediated hemolysis of rabbit erythrocytes with 10 % NHS or 10 % depleted serum. A$_{414}$ nm, absorbance at 414 nm. The mean values out of three separate experiments are shown and error bars correspond to SD. B, C5b-9 deposition via the alternative (20 %), lectin (1 %) and classical (1 %) pathways. Activation was recorded by following TCC assembly using an anti-C5b-9 mAb and HRP-conjugated rabbit anti-mouse pAb.

Complement activation assays

Normal human serum (NHS) obtained from five healthy volunteers upon informed consent was pooled. Human serum was depleted from Factor H/FHL-1/CFHR1 by affinity chromatography using Factor H antiserum coupled to a mix of protein A-Sepharose and protein G-Sepharose (GE Healthcare) overnight at 4°C as described previously (1). To demonstrate that this depleted serum i.e. HSΔFH/FHL-1/CFHR1 was complement active, complement activity was assayed in a hemolytic assay using rabbit erythrocytes (RabbitER) (Rockland, BioTrend Chemikalien GmbH, Köln, Germany). RabbitER (10$^8$/ml) were incubated with HSΔFactor H/FHL-1/CFHR1 (10 %) diluted in MgEGTA buffer (20 mM HEPES, 144 mM NaCl, 7 mM MgCl$_2$, and 10 mM EGTA, pH 7.4). All incubations were performed in a final volume of 100 μl. After incubation for 15 min at 37 °C, intact
erythrocytes were sedimented by centrifugation and erythrocytes lysis was determined by measuring the release of hemoglobin at 414 nm.

To demonstrate that all three complement pathways were active after depletion of the regulators an ELISA based activation assay was used. The alternative, lectin or classical pathways were activated separately by specific activators and following incubation TCC (C5b-9) deposition was assayed (2). Microtiter plates were coated with LPS (10 μg/ml) (activation of the alternative pathway), mannan (100 μg/ml) (activation of the lectin pathway) or IgM (2 μg/ml) (activation of the classical pathway) over night at 4°C, washed four times with PBS-Tween and blocked for 1 h at 37°C with PBS containing 2 % BSA. NHS was diluted in MgEGTA buffer (alternative path way) or GVB++ buffer (Complement Technology) (classical/lectin pathways), added to the microtiter plates with activators and incubated for 1 h at 37°C. Heat-inactivated NHS (HiNHS) (56°C, 30 min) was used as a negative control. After washings, complement activation was assayed by determining TCC deposition with a mouse anti-human C5b-9 mAb (Dako) and HRP-conjugated rabbit anti-mouse pAbs. The reaction was developed with 1,2-phenylenediamine dihydrochloride (OPD; Dako). The absorbance was measured at 492 nm.

**Factor H/FHL-1/CFHR1-depleted human serum is complement active**

In order to determine that the depleted serum maintains complement activity, this activity was determined by two independent and separate approaches. First the hemolytic activity of the depleted serum was tested using rabbit erythrocytes (RabbitER). RabbitER are lysed when incubated in NHS (Fig. 2A, *column 2*). Similar, RabbitER incubated in HSAΔFactor H/FHL-1/CFHR1 were lysed, thus demonstrating that the serum complement was still active (Fig. 2A, *column 3*). However, the lytic activity of the depleted serum is lower as compared to the NHS (∼40 %). B, In addition, complement activity of the depleted serum was assayed for all three
complement pathways using a ELISA based activation assay. Each activation pathway, i.e. the alternative, the lectin or the classical complement pathway was specifically activated and after incubation TCC (C5b-9) deposition was assayed. All three pathways were still active in the depleted serum. However, when activated via the alternative pathway the activity of the serum was 30 % reduced compared to NHS (Fig. 2B). Similarly, the classical pathway activity in HS\(\Delta Factor H/FHL-1/CFHR1\) was reduced by 24 % and the lectin pathway was not affected.