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The Streptococcal Lipoprotein Rotamase A (SlrA) Is a Functional Peptidyl-prolyl Isomerase Involved in Pneumococcal Colonization*

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Peter W. M. Hermans[‡1,2], Peter V. Adrian[§1,3], Christa Albert[¶], Silvia Estevão[§], Theo Hoogenboezem[§], Ingrid H. T. Luijendijk[§], Thilo Kamphausen[‖4], and Sven Hammerschmidt[¶5]

From the [‡]Department of Pediatrics, University Medical Center St. Radboud, 6500 HB Nijmegen, The Netherlands, the [§]Department of Pediatrics, Erasmus Medical Center, Sophia Children's Hospital, 3000 DR Rotterdam, The Netherlands, the [¶]Research Center for Infectious Diseases, University of Würzburg, 97070 Würzburg, Germany, and the [‖]Max-Planck-Research Unit for Enzymology of Protein Folding, 06120 Halle, Germany

Streptococcus pneumoniae **expresses two surface-exposed lipoproteins, PpmA and SlrA, which share homology with distinct families of peptidyl-prolyl isomerases (PPIases). In this study, we demonstrated for the first time that the lipoprotein cyclophilin, SlrA, can catalyze the *cis-trans* isomerization of proline containing tetrapeptides and that SlrA contributes to pneumococcal colonization. The substrate specificity of SlrA is typical for prokaryotic and eukaryotic cyclophilins, with Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (*p*NA) being the most rapidly catalyzed substrate. In a mouse pneumonia model the *slrA* knock-out D39Δ*slrA* did not cause significant differences in the survival times of mice compared with the isogenic wild-type strain. In contrast, a detailed analysis of bacterial outgrowth over time in the nasopharynx, airways, lungs, blood, and spleen showed a rapid elimination of *slrA* mutants from the upper airways but did not reveal significant differences in the lungs, blood, and spleen. These results suggested that SlrA is involved in colonization but does not contribute significantly to invasive pneumococcal disease. In cell culture infection experiments, the absence of SlrA impaired adherence to pneumococcal disease-specific epithelial and endothelial non-professional cell lines. Adherence of the *slrA* mutant could not be restored by exogenously added SlrA. Strikingly, deficiency in SlrA did not reduce binding activity to host target proteins, but resulted in enhanced uptake by professional phagocytes. In conclusion, SlrA is a functional, cyclophilin-type PPIase and contributes to pneumococcal virulence in the first stage of infection, namely, colonization of the upper airways, most likely by modulating the biological function of important virulence proteins.**

Peptidyl-prolyl *cis*/*trans* isomerases (PPIases)[6] are ubiquitous foldases, which accelerate the rate-limiting *cis-trans* or *trans-cis* conformational changes at Xaa-Pro bonds during protein folding in both eukaryotes and prokaryotes. There are three distinct families within the enzyme class of PPIases (EC 5.2.1.8): the cyclophilins, which bind the immunosuppressant cyclosporin A, the FK506-binding proteins, and the parvulins. The classical PPIase such as rotamase A of *Escherichia coli* is a member of the cyclophilin family (1, 2). The FK506-binding proteins all have high affinity for the immunosuppressant drug FK506 (3, 4). The ubiquitous bacterial trigger factor (5, 6) and the macrophage infectivity potentiator (Mip) protein of *Legionella pneumophila* (7) belong to the FK506-binding proteins. The parvulin family resembles enzymes such as parvulin, SurA, and PrsA. The *E. coli* parvulin 10 with its 92 amino acids is one of the smallest known PPIase enzymes and represents the prototype of this family (8). SurA contributes to the assembly of outer membrane proteins in Gram-negative bacteria (9). PrsA is considered to be involved in enzyme secretion and activation in Gram-positive bacteria (10–12).

Streptococcus pneumoniae (the pneumococcus) is a frequent colonizer of the human host. During colonization, the bacterium adheres to the nasopharyngeal epithelium, a dynamic process that requires various adhesion molecules such as the major pneumococcal adhesin, which interacts in a human specific manner with the secretory component of the polymeric Ig receptor (13–15). Besides colonization, the pneumococcus is a major cause of both invasive diseases such as meningitis, septicemia, and pneumonia, and non-invasive diseases such as otitis media and sinusitis (16–18). To cause disease, the pneumococcus has to migrate from the nasopharynx and disseminate into other parts of the human host, such as the lungs, blood, or meninges. The contents of the bacterial surface play an important role in the different disease processes. Data mining of sequenced *S. pneumoniae* genomes identified four putative PPIases, a cytoplasmic cyclophilin homologue, trigger factor, the ubiquitous bacterial FK506-binding proteins, and two lipoproteins, which resembled homology with distinct families of PPIases. Of the lipoproteins, the putative proteinase maturation protein A (PpmA) was homologous with the parvulin family (19, 20), whereas the streptococcal lipoprotein rotamase A (SlrA) shared homology with the cyclophilins (21).

There has been growing evidence that PPIases contribute to bacterial virulence. For example, the Mip protein of *L. pneumophila* is involved in

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[1] Both authors contributed equally to this work.
[2] To whom correspondence may be addressed: Laboratory of Pediatric Infectious Diseases, University Medical Center St. Radboud, P. O. Box 9101 (internal post 184), 6500 HB Nijmegen, The Netherlands, Tel.: 31-24-3666406; Fax: 31-24-3616428; E-mail: p.hermans@cukz.umcn.nl.
[3] Present address: University of the Witwatersrand, Johannesburg, South Africa.
[4] Present address: Strathmann Biotec AG, Bovenau, Germany.
[5] To whom correspondence may be addressed: Röntgenring 11, Würzburg, D-97070, Germany. Tel.: 49-931-31-2153; Fax: 49-931-31-2578; E-mail: s.hammerschmidt@mail.uni-wuerzburg.de.

[6] The abbreviations used are: PPIase, peptidyl-prolyl isomerase; CsA, cyclosporin A; SlrA, streptococcal lipoprotein rotamase A; PpmA, putative proteinase maturation protein A; Mip, macrophage inhibitor potentiator; Cps, capsular polysaccharide; pNA, *p*-nitroanilide; PBS, phosphate-buffered saline; SC, secretory component.

entry of host cells and intracellular replication (22–25), and the trigger factor of *Streptococcus pyogenes* is considered to be responsible for secretion of several virulence factors (26).

We, therefore, investigated whether PPIases of *S. pneumoniae*, in particular those that are surface-located, contribute to bacterial virulence. In this study, the protease-coupled PPIase assay indicated PPIase activity and substrate specificity for SlrA. More importantly, the contribution of SlrA to virulence was demonstrated *in vivo* using a murine model for pneumococcal pneumonia. Cell culture infection experiments indicated that SlrA expression interferes with non-tissue specific cellular adhesion but probably not invasion. Finally, expression of SlrA is involved in protection of pneumococci against phagocytosis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture Conditions, and Protein Purification—*S. pneumoniae* were cultured on blood agar plates (Oxoid, Wesel, Germany) at 37 °C and 5% CO_2, or cultured in Todd-Hewitt broth (Roth, Karlsruhe, Germany) supplemented with 0.5% yeast extract (THY) to mid-log phase. Strain validation involved Optochin sensitivity (Difco, Detroit, MI) and the Quellung reaction. D39 (Cps^+ serotype 2), which *in vitro* shows only low levels of adherence (27), was employed in mouse infection experiments, whereas the NCTC10319 (Cps^+ serotype 35A) was used in cell culture-based infection experiments. The non-mouse virulent NCTC10319 adheres efficiently to host cells, and with respect to the genetic background, virulence factors such as pneumolysin, choline-binding proteins, and PavA are identically expressed compared with D39 (28, 29). During co-infection colonization studies, the *rpsL* gene encoding a streptomycin-resistant mutant of the ribosomal protein S12 was used to transform D39. Streptomycin-resistant D39 transformants (D39::*rpsL*) were selected in THY broth containing 100 μg ml^{-1} streptomycin, and a single transformant clone was invariably used in our animal experiments. To maintain virulence, bacteria were animal passaged prior to use in infections, as described previously (30). Isogenic mutants lacking the genes encoding *slrA* and *ppmA* were selected on blood agar with 1 μg ml^{-1} of erythromycin and 20 μg ml^{-1} of trimethoprim, respectively. Functional activity of the pneumolysin was assessed using the hemolysis assay (31). *E. coli* BL21(DE3) (Stratagene) was used as the host strain for recombinant SlrA and PpmA expression, and cultured at 30 °C on Luria-Bertani (LB) agar or LB broth containing 100 μg ml^{-1} of ampicillin and 50 μg ml^{-1} of kanamycin.

Construction of Pneumococcal Mutants—The genes encoding *slrA* (accession number spr0679) and *ppmA* (accession number spr0884) were deleted from the parental strains D39 and NCTC10319 by in-frame insertion deletion mutagenesis using the resistance cassettes *ermAM* (32), and *dfr13* (33), encoding resistance to erythromycin and trimethoprim, respectively. *SlrA* and *ppmA* with 1000 bp of upstream and downstream flanking sequences were amplified from chromosomal DNA from the D39 strain. The PCR products were cloned into pBlueScript KS+ and transformed to *E. coli* DH5α (Stratagene). The gene regions of *slrA* and *ppmA* were deleted from their respective plasmids by performing an inverse PCR with primers that amplify the flanking sequences and pBlueScript KS+. These amplicons were ligated with the PCR-amplified *ermAM* and *dfr13*, respectively, and transformed to *E. coli* DH5α (Stratagene). The primers covering the 5′-flanking sequence of *slrA* and *ppmA* were designed to include NdeI restriction sites that overlap the start codons of these genes. Similarly, *ermAM* and *dfr13* were amplified with NdeI sites at the start codon, to ensure that resistance cassettes were cloned in the correct orientation, and were transcribed from the host promoters, starting from the ATG start codon. These erythromycin- and trimethoprim-resistant knock-out constructs were used as template DNA to generate linear PCR fragments containing the flanking regions of *slrA* and *ppmA*, and resistance genes *ermAM* and *dfr13*, respectively. The amplified DNA was used to delete the *slrA* and *ppmA* genes in strains D39 (serotype 2) and NCTC10319 (serotype 35A) by natural transformation as described previously (19). The *slrA* and *ppmA* fragments that were removed were of equal length to the inserted cassettes to eliminate the possibility of any polar effects. Furthermore, analysis of the flanking sequences of *slrA* and *ppmA* indicated that these genes had their own promoters, and were not part of an operon that could be affected by these gene modifications. Transcription profiling using genomic microarrays of the wild-type and mutant strains grown to mid-log phase, in THY broth, indicated that with the exception of the mutant alleles, no other significant transcriptional changes were observed (data not shown).

Protease-coupled PPIase Assay—The PPIase activity and substrate specificity of SlrA and PpmA were determined using the protease-coupled assay as published previously (34, 35). Briefly, the *cis* to *trans* isomerization of various substrates with the consensus sequence Suc-Ala-Xaa-Pro-Phe-*p*NA (20 mg ml^{-1} in Me_2SO) was followed at 10 °C and a wavelength of 390 nm by the *trans*-specific proteolytic cleavage of the Phe-*p*NA bond. Xaa represents the varied amino acid residues analyzed at this position of the substrate. A reference signal recorded at 510 nm is expected not to show a signal change during the measurements. To analyze the inhibition by CsA or specific antisera recognizing SlrA or PpmA, respectively, the proteins were preincubated in buffer and with the inhibitor for 5 min, and the measurements were started by addition of substrate and protease (chymotrypsin). The inhibition constant K_i was calculated with SigmaPlot software using the formula for slow tight binding inhibitors. The enzymatic activity k_{enz} was calculated by subtracting the uncatalyzed reaction from the observed reaction within the measurement (36, 37).

Mouse Pneumonia Model—To study survival times of mice in a mouse pneumonia model, pneumococcal cultures of $A_{600} = 0.3$ were diluted in phosphate-buffered saline (PBS) to a final concentration of 10^6 colony-forming units/50 μl. Nine-week-old CD-1 mice (Harlan, Horst, Netherlands) were lightly anesthetized with 2.5% (v/v) isoflurane over oxygen (1.5 liter min^{-1}), administered with a calibrated vaporizer, and anesthesia was confirmed by observing no pinch reflex reaction. Once anesthetized the animals were scuffed, with the nose held upright, and 50 μl of pneumococci or pneumococcal mutants were introduced intranasally by adding a series of small droplets of the inoculum into the nostril for the mice to involuntarily inhale. After inoculation, mice were held upright for 20 s, and then laid on their backs until recovery. Signs of disease were closely monitored until the animals were deemed moribund (38) when they were sacrificed by cervical dislocation. Mice displaying no signs of illness by 336 h were considered to have survived the infection. To study bacterial outgrowth during infection, mice were inoculated, as described above, and sacrificed by cervical dislocation at 0, 6, 12, and 24 h post-challenge (5 animals per time point). Blood samples were obtained by cardiac puncture. The nasopharynx was washed using a modified method of Wu *et al.* (39). The trachea was exposed and clamped and 2 ml of sterile PBS was passed through the nasopharynx via a 16-gauge non-pyrogenic angiocath (F. Baker Scientific, Runcorn, UK). Bronchi alveolar lavage, lung, and spleen tissue sampling were then carried out as previously described (38). Bacterial loads were determined by plating out 10-fold dilutions onto blood agar plates. For bacterial co-infection experiments with streptomycin-resistant D39 (D39::*rpsL*) and D39Δ*slrA*, mice were inoculated as described above, with 5×10^5 colony-forming units of each bacterial strain in 50 μl of PBS. Sampling occurred as described above, and bacterial loads were counted on

blood agar containing either 1 μg ml^{-1} of erythromycin or 100 μg ml^{-1} of streptomycin. All experiments were carried out in accordance with the regulations laid out by the Dutch animal ethics committee.

Recombinant Proteins and Antiserum—The *slrA* gene (accession number spr0679) was amplified by PCR from *S. pneumoniae* D39 with primers 5′-TTTACTGCATATGCACCATCACCATCACCATAGC-AGCGTCCAACGCAGT-3′ and 5′-CATTAGGATCCAATCGCT-GGGGAAGTG-3′, which incorporated flanking NdeI and BamHI restriction sites, and a N-terminal His_6 tag. The *ppmA* gene (accession number spr0884) was amplified by PCR from *S. pneumoniae* D39 with primers 5′-CCATGGCTAGCCACCATCAC CATCACCATTC-GAAAGGGTCAGAAGGTGC-3′ and 5′-TCATGGATCCGGAC-TATTCGTTTGATGTAC-3′, which incorporated flanking NheI and BamHI restriction sites, and a N-terminal His_6 tag. The amplified DNAs were cloned into a similarly digested pET11a expression vector (Stratagene) and electrotransformed into *E. coli* BL21(DE3). The recombinant proteins were purified by Ni^+ affinity chromatography with the HisTrap Kit (Amersham Biosciences) according to the manufacturer's recommendations. The purified recombinant protein was dialyzed against 10 mM HEPES buffer, pH 7.5, freeze dried, and stored at −20 °C. The integrity of the DNA inserts was verified by sequence analysis using ABI Prism dye terminator cycle sequencing (Applied Biosystems).

Polyclonal anti-PpmA and anti-SlrA antiserum was raised in rabbits by routine immunogenic procedures (Eurogentec, Seraing, Belgium). Prior to its use in cell infection inhibition experiments, anti-PpmA-, anti-SlrA-, and preimmune serum were protein A purified using Sepharose 4B affinity chromatography (Amersham Biosciences). Concentrations of the antibodies were 1.5 mg ml^{-1} for anti-SlrA IgG and 2.0 mg ml^{-1} for preimmune IgG, respectively.

Cell Lines and Cell Culture—The human lung alveolar carcinoma epithelial cell line A549 (ATCC CCL-185; type II pneumocytes) and the murine J774A.1 macrophage-like cell line (ATCC TIB 67) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin G (100 IU ml^{-1}), and streptomycin (100 μg ml^{-1}) (all from PAA Laboratories, Coelbe, Germany). The human nasopharyngeal epithelial cell line Detroit 562 (human pharynx carcinoma; ATCC CCL 138) was cultured in RPMI 1640 (PAA Laboratories) supplemented with 10% fetal calf serum, 2 mM glutamine, and 1 mM sodium pyruvate. Human umbilical vein-derived endothelial cells (Cambrex) were cultured in endothelial cell growth medium 2 (PromoCell, Heidelberg, Germany) in the presence of the SupplementMix (PromoCell). All cells were cultured at 37 °C and 5% CO_2.

Adherence and Internalization Assays—For adherence and phagocytosis experiments, 5×10^4 cells per well were seeded in antibiotic-free medium on 24-well tissue culture plates (Greiner, Germany) and incubated for 48 h. For immunofluorescence studies cells were seeded on glass coverslips (diameter, 12 mm) at a cell density of 5×10^4 per well. Confluent cell layers with $\sim 2 \times 10^5$ cells were washed prior to infection with medium and the cells were infected with pneumococci in 500 μl of Dulbecco's modified Eagle's medium with HEPES (once; PAA Laboratories) and 1% fetal calf serum per well with a multiplicity of infection of 100 pneumococci per cell at 37 °C under 5% CO_2. In complementation or blocking experiments, infections were either carried out in the presence of different amounts of rSlrA (1 μg/well to 20 μg/well), or 5×10^6 wild-type pneumococci or isogenic *slrA* mutants were preincubated with 5 or 10 μg of polyclonal anti-SlrA IgG or preimmune IgG antibodies in a total volume of 100 μl prior to infection. The polyclonal anti-SlrA IgG (2, 5, or 10% (w/v)) did not affect growth cultures as indicated by identical growth curves in the presence or absence of anti-SlrA IgG (data not shown).

The number of viable intracellular pneumococci after phagocytosis by macrophages or invasion of epithelial as well as endothelial cells was quantified by the antibiotic protection assay as described previously (29). Briefly, the infected cell monolayers were washed with Dulbecco's modified Eagle's medium with HEPES to remove unbound pneumococci and incubated with 1 ml/well of Dulbecco's modified Eagle's medium with HEPES containing 100 μg of gentamicin and 100 units of penicillin G to kill extracellular bacteria. The cells were washed again and release of intracellular pneumococci was conducted by a saponin-mediated lysis (1% w/v) of the cells. The released intracellular pneumococci per well were enumerated by plating serial dilutions on blood-agar plates.

Determination of Adherence by Double Immunofluorescence—Infected cells were washed three times with PBS to remove unbound bacteria and the cells and bacteria were fixed for 20 min on glass coverslips with 3.7% paraformaldehyde. The fixed samples were washed with PBS and nonspecific binding sites were blocked for 30 min with 10% fetal calf serum. The blocking buffer was removed and samples were incubated for 45 min with a rabbit anti-pneumococcal antiserum (1:100), followed by a 30-min incubation with Alexa Fluor 488-labeled goat anti-rabbit Ig (MoBiTec, Göttingen, Germany) to stain extracellular pneumococci. The reactivity of the anti-pneumococcal antiserum was described previously (28, 29). In some experiments the intracellular bacteria were enumerated by double immunofluorescence. Therefore, the samples were washed with PBS and the cells were permeabilized for 5 min with 0.1% Triton X-100. Thereafter, the samples were incubated with anti-pneumococcal antibody, washed, and incubated with Alexa Fluor 568-labeled goat anti-rabbit Ig (MoBiTec) to stain intra- and extracellular bacteria. After he final washing steps with PBS, the coverslips were embedded "upside down" in Moviol, sealed with nail polish, and stored at 4 °C. All antibody incubations were performed at room temperature in PBS. Numbers of extracellular pneumococci were determined using a fluorescence microscope (Zeiss Axioskop) and image acquisition was carried out using a confocal laser scanning microscope (CLSM) and the CLSM software (Zeiss). Each experiment was repeated at least three times and results were expressed as mean ± S.D.

Binding of Pneumococci to Immobilized Host Proteins and Extracellular Matrix—96-Well microtiter plates (polystyrene surface) were coated with human proteins (2.5 μg) such as fibronectin, fibrinogen, laminin, type IV collagen IV, secretory component, and lactoferrin, as well as reconstituted basement membrane (Matrigel®; BD Biosciences) at 4 °C overnight. The proteins were purchased from commercial suppliers (Calbiochem, Sigma, and Roche). The surface of the wells was subsequently blocked with 1% bovine serum albumin for at least 3 h at room temperature. Labeling of the bacteria with fluorescein isothiocyanate was performed as described (40). Extensively washed fluorescein isothiocyanate-labeled wild-type or *slrA*-deficient pneumococci (2.5×10^7) were added to the wells and incubated for 1 h at 37 °C to allow binding. Fluorescence was measured at 485/538 nm (excitation/emission) using a Fluoroskan Ascent (ThermoLabsystems).

Statistical Analysis—Survival times were compared using Mann-Whitney U analysis. Results from bacteriology time courses were compared using one-way analysis of variance with Scheffe's post-hoc test. Bacteriology results below the detection limits for the viable counting assay (log 1.92 per ml of blood or log 0.92 per ml of lung or nasopharyngeal samples) were ascribed a value just below the detection limit (log 1.91 or 0.91). Comparisons of bacterial loads between bacterial strains were carried out using multiple unpaired Student's *t* tests with Bonferroni correction. In D39:*rpsL* and D39Δ*slrA* co-infection studies *in vitro* and *in vivo* the data were mathematically corrected to account for the

differences in count in the initial inoculum. Virulence data were expressed as mean ± S.E., whereas adherence and invasion data were expressed as mean ± S.D. Differences in virulence, adherence, and invasion were analyzed by the two-tailed unpaired Student's *t* test. In all analyzes, a p value of <0.05 was considered statistically significant.

RESULTS

SlrA Is a Functional, Cyclophilin-type PPIase—The PPIase activity of both surface-exposed lipoproteins, PpmA and SlrA, was measured using the protease-coupled assay. PpmA showed no significant PPIase activity (data not shown). In contrast, SlrA clearly demonstrated PPIase activity (Table 1). The alignment of SlrA and the well characterized human prototypic cyclophilin, hCyp18, demonstrated a sequence similarity over full sequences of 27% and a sequence identity of 20% (Fig. 1). Although the sequence homology between SlrA and hCyp18 is low with respect to homologous proteins, the enzymatic activity was only 1 order of magnitude lower compared with hCyp18. The substrate specificity and the relative rate of SlrA activity were also determined. The highest k_{cat}/K_m was demonstrated for the substrate peptide where alanine preceded the proline residue (Table 1). Comparison of the substrate specificities showed that it differed from hCyp18 in that the substrate specificity was more closely related to other *prokaryotic* cyclophilins like *Eco*CypB (41), *Streptomyces antibioticus San*Cyp18, or *L. pneumophila Lp*Cyp18 (37). In addition, the substrate specificity was not highly restricted to alanine preceding the proline residue (Table 1). Although the general sequence identity is below 30%, the k_{cat}/K_m for alanine substrate was high, *i.e.* 1.1×10^6 M^{-1} s^{-1}. This can be explained by the fact that only two of the 13 amino acids (85% identity), which have been reviewed to be catalytically relevant, are exchanged (42) (Fig. 1). Within the alignment of hCyp18 and SlrA, the SlrA protein shows four regions where the amino acid sequence is extended by at least 6 amino acids (Fig. 1). Assuming that a three-dimensional fold similar to hCyp18 is required for the PPIase activity, three of these extensions are very close together on the surface of the SlrA protein, widening a secondary turn element of the active. Only the third extension is present in *Eco*Cyp18, *San*Cyp18, and *Lp*Cyp18, whereas the first and second are missing (37).

The substrate Suc-Ala-Ala-Pro-Phe-*p*NA was used to analyze the inhibition of SlrA by cyclosporin A (CsA). The inhibition constant K_i was 880 ± 120 nM (Fig. 2), which is more than 100 times higher than hCyp18 (5 nM), but 2 times more effective compared with other *prokaryotic* cyclophilins, whose K_i is reported in the micromolar range (37). The antiserum generated against SlrA was analyzed for inhibitory effects on SlrA PPIase activity with Suc-Ala-Ala-Pro-Phe-*p*NA as substrate. In the presence of rabbit anti-SlrA IgG, the PPIase activity was reduced by 90% (data not shown).

SlrA Is Involved in Bacterial Virulence in Vivo—The gene encoding SlrA in the parental strain D39 (serotype 2) and NCTC10319 (serotype 35A) were inactivated using insertion deletion mutagenesis. Replacement of *slrA* with *ermAM* was confirmed by nucleotide sequence analysis. The erythromycin resistance cassette was stable, and no loss of resistance occurred following growth *in vitro* and *in vivo*. The absence of protein expression was demonstrated by immunoblot analysis using anti-SlrA monospecific rabbit serum (Fig. 3). The mutants were identical to wild-type strains with respect to cell morphology, colony morphology, growth rate *in vitro* as determined by measurement of optical density, and colony forming units over time, and the rate of autolysis during stationary phase. In addition, there were no differences between the *slrA* mutants and the wild-type strains with respect to sensitivity to antibiotics, detergents, pH, temperature, and hemolysis caused by pneumolysin, which represents a key virulence factor (Ref. 43 and data not shown). To determine the effect of inactivation of *slrA* on virulence in a pneumonia model, mice were challenged with 10^6 bacteria via the intranasal route with strain D39 and the D39Δ*slrA* mutant, respectively. There were no significant differences ($p = 0.19$) in the survival times between groups of mice ($n = 10$) challenged with D39 and mutants (data not shown). A more detailed investigation was carried out on rates of bacterial outgrowth over time in the nasopharynx, airways, lungs, blood, and spleen. Using groups of $n = 5$ per time point, no significant differences between the rate of bacterial outgrowth at the various sites in mice challenged with wild-type D39 and D39Δ*slrA* mutants at any of the sites was measured. Whereas there was a trend toward more rapid elimination of the mutant strain from the nasopharynx and airways,

TABLE 1

Substrate specificity and relative rate of SlrA compared with hCyp18

PPIase activity was determined at 390 nm with the isomer-specific proteolytic test at 10 °C in 35 mM HEPES, pH 7.5. K_{cat}/K_m is given in mM^{-1} s^{-1}, and the relative rate in percentage with the PPIase activity for the substrate Suc-Ala-Ala-Pro-Phe-*p*NA set at 100%. -Xaa- represents various amino acids of substrates with the general sequence Suc-Ala-Xaa-Pro-Phe-*p*NA. The hCyp18 substrate specificity has been determined previously (36).

Substrate(-Xaa- =)	SlrA, K_{cat}/K_m	hCyp18, K_{cat}/K_m
	mM^{-1} s^{-1}	
-Ala-	1,144 ± 38 (100%)	17,600 (100%)
-Phe-	397 ± 4 (35%)	2,700 (15%)
-Gly-	421 ± 21 (37%)	1,800 (10%)
-Val-	866 ± 68 (76%)	5,400 (31%)
-Leu-	785 ± 79 (67%)	5,000 (29%)
-Gln-	308 ± 45 (27%)	1,000 (5,7%)
-Glu-	147 ± 37 (13%)	4,300 (24%)

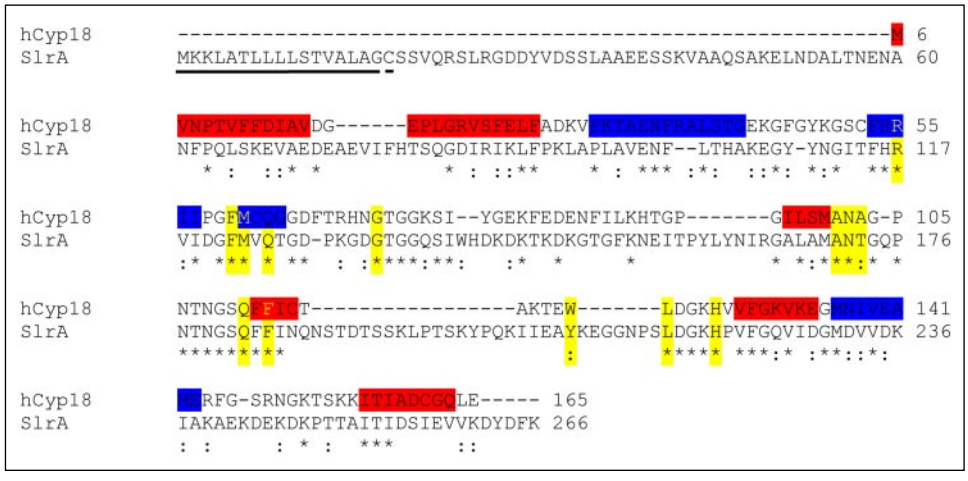

FIGURE 1. **Protein sequence alignment between SlrA and hCyp18.** The predicted signal sequence and lipid binding site of SlrA is *underlined*. Identical amino acids are marked with *asterisks*, similar amino acids with *dots*; gaps are depicted with *horizontal lines*. The amino acids of the catalytic center of hCyp18 are marked in *yellow*, the secondary structure elements of hCyp18 are indicated in *red* (β-sheet), and *blue* (α-helix), as analyzed in the crystal structure of hCyp18 (2).

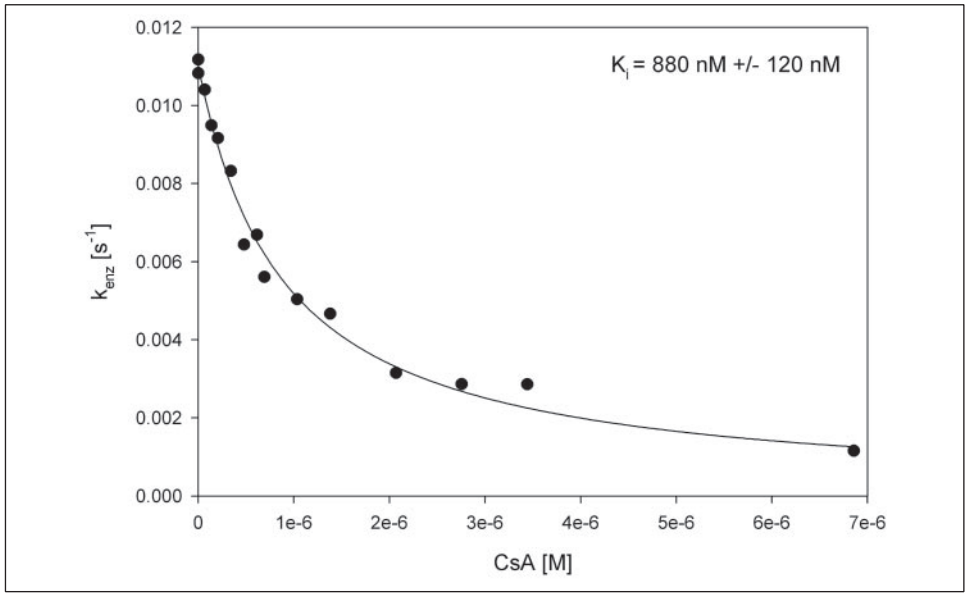

FIGURE 2. **Inhibition of SlrA PPIase activity by CsA.** The substrate Suc-Ala-Ala-Pro-Phe-*p*NA was used. The inhibition constant K_i was calculated with SigmaPlot software using the formula for slow tight binding inhibitors. The enzymatic activity k_{enz} was calculated by subtracting the uncatalyzed reaction from the observed reaction within the measurement (36, 37).

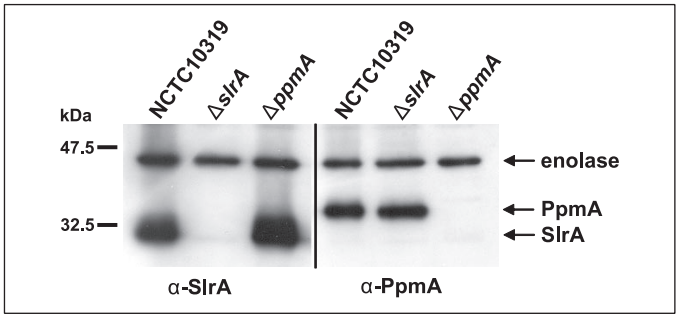

FIGURE 3. **Immunoblot analysis of pneumococcal strain NCTC10319 and its isogenic Δ*slrA* and Δ*ppmA* mutants.** The results revealed no cross-reaction between anti-SlrA IgG and PpmA protein or anti-PpmA and SlrA protein.

because of the variation of sampling between individual mice, the results were not significant (data not shown).

To reduce the variance in the experimental setup caused by variation between individual mice, inoculation efficiency, and sample collection, mice were co-infected with equal numbers (5×10^5 colony forming units) of both D39Δ*slrA* mutant and a streptomycin-resistant D39 parent strain. The percentage change in colony forming units over time of the mutant, relative to the D39 parent strain within each individual mouse was then calculated, and a clear deficiency in the ability of D39Δ*slrA* to colonize the nasopharynx and airways could be demonstrated (Fig. 4, *A* and *B*). Within 6 h of colonization by D39Δ*slrA* relative to wild-type, D39 was decreased by 70 and 50% in the nasopharynx ($p < 0.001$) and airways ($p < 0.05$), respectively. The ratios of wild-type D39 and D39Δ*slrA* that were recovered from the lungs, bloodstream, and spleen after 12 and 24 h were highly variable, and no significant differences were measured. This suggests that upon contact with the lung tissue, the *slrA*-negative mutant and wild-type strain were equal in their ability to cause invasive disease. The high variability in the mutant *versus* wild-type ratios measured in individual mice suggest there is a population "bottleneck" effect, caused by a small number of organisms breaking through the host immune defenses, followed by outgrowth of individual organisms in the lung tissue and blood.

Contribution of SlrA to Pneumococcal Adherence in Vitro—As SlrA might affect virulence of pneumococci by its contribution to adherence, cell culture infections with human epithelial and endothelial cells were performed. The serotype 35A strain NCTC10319 and its isogenic *slrA* mutant were assessed by double immunofluorescence microscopy for their ability to adhere to and invade Detroit cells, A549 cells, and human umbilical vein-derived endothelial cells. These cell lines are used as models for nasopharyngeal colonization, pneumococci adherence to alveolar epithelial cell, and pneumococci interaction with the endothelium, respectively. Adherence of the *slrA* knock-out strain to epithelial cell lines Detroit 562 and A549, respectively, or to human umbilical vein-derived endothelial cells, evaluated in kinetic experiments after 2, 3, or 4 h was significantly reduced at all time points compared with the adherence of the corresponding wild-type strain (Fig. 5, *A* and *B*). The adherence of the parental strain and the isogenic *slrA* mutant was elevated over time, however, the increase of adherence over time was significantly lower for the *slrA* knock-out (data not shown).

A quantitative assessment of pneumococci internalized by epithelial or endothelial cells was performed by employing the antibiotic protection assay. The results revealed a substantial reduction of recovered intracellular bacteria for the *slrA* mutant compared with the isogenic wild-type (Fig. 5*C*). The degree of reduction was equivalent to the reduction in adherence determined by fluorescence microscopy.

To investigate whether SlrA has a direct effect on adherence of pneumococci, the cell culture infection experiments were conducted in the presence of different concentrations of rSlrA. Adherence of the wild-type strain and the mutant was not affected by co-cultivation with the rSlrA protein as indicated by immunofluorescence. To exclude the possibility that SlrA directly interacts with epithelial or endothelial cells, SlrA-coated microspheres were incubated with these cells as described recently for SpsA-coated microspheres (28). The results did not show binding of SlrA-coated microspheres to eukaryotic cells (data not shown). PPIase activity was inhibited by anti-SlrA IgG. In contrast, the presence of anti-SlrA in infection experiments or the pre-treatment of pneumococci with anti-SlrA IgG did not significantly impair pneumococcal adherence (data not shown).

The reduced efficiency of adherence and uptake of the *slrA* mutant was cell-type independent, and rSlrA protein was neither able to complement the pneumococcal deficiency in SlrA nor showed a direct interaction with host cells, suggesting that SlrA affects virulence proteins with key biological functions at different stages of the infection, in particular during colonization. In conclusion, the cell culture infections

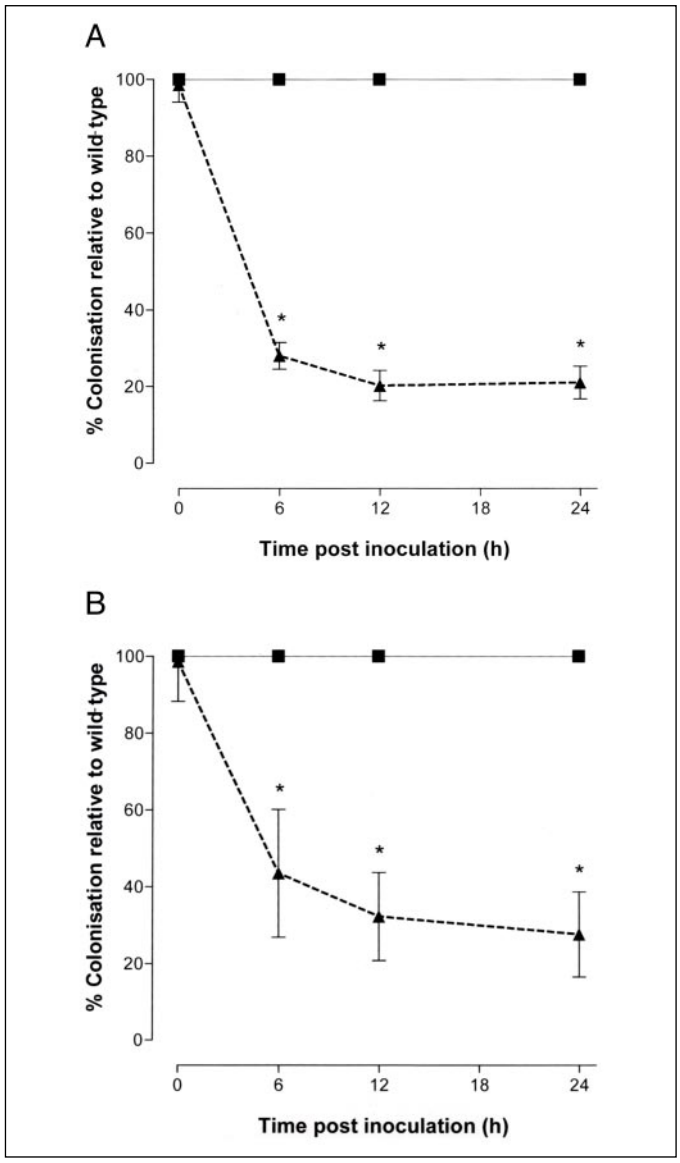

FIGURE 4. **Intranasal challenge of mice with streptomycin-resistant pneumococcal strain D39 (D39:*rpsL*) and D39Δ*slrA*.** Four groups of 5 mice each were co-infected with 5×10^5 colony-forming units of each bacterial strain. Bacterial loads were counted for D39:*rpsL* (*squares*) and D39Δ*slrA* (*triangles*) at 0 (group 1), 6 (group 2), 12 (group 3), and 24 h (group 4) post-inoculation at the nasopharynx (*A*) and airways (*B*). *Vertical lines* depict standard deviation. Statistical significance ($p < 0.05$) is marked with *asterisks*.

suggested an important and indirect function of SlrA for adherence, but most likely not for subsequent invasion, because invasion was equally influenced as adherence was.

Effects of SlrA on Pneumococcal Host Protein Binding Activities—*S. pneumoniae* binds to various host proteins derived from the extracellular matrix or serum (44–49) and via SpsA to the secretory component (SC) of the human polymeric Ig receptor that is expressed on mucosal epithelial cells (13, 15, 28). To assess whether deficiency in *slrA* affects pneumococcal surface proteins interacting with host proteins or cellular receptors, binding of pneumococcal wild-type and its isogenic *slrA* mutant to immobilized extracellular matrix proteins or reconstituted basement membrane (Matrigel®) was investigated. The results did not reveal significant differences in the ability of the *slrA* mutant to bind to SC, type IV collagen, fibronectin, fibrinogen, laminin, lactoferrin, or Matrigel (Fig. 6). These data indicated that the surface expression of pneumococcal adhesins for SC, lactoferrin, and adhesins involved in binding to the other used proteins of the extracellular matrix is not affected by disruption of the *slrA* gene. Because the SC-binding protein SpsA and lactoferrin-binding protein PspA belong to the family of choline-binding proteins, these data further indicated that SlrA has no effect on expression and functional activity of pneumococcal choline-binding proteins.

SlrA Affects Phagocytosis of S. pneumoniae by Macrophages—To assess the effect of SlrA on pneumococcal uptake in professional phagocytes, pneumococci and isogenic Δ*slrA* mutants of serotype 35A strain NCTC10319 were incubated for 1 h with J774 macrophages. After antibiotic killing of the extracellular bacteria, the number of internalized viable wild-type pneumococci and corresponding isogenic mutants were monitored by quantitative platings. The results revealed significantly elevated numbers of internalized Δ*slrA* mutants of NCTC10319 (Fig. 7). These differences were confirmed in the double immunofluorescence (Fig. 5*B*). The immunofluorescence showed a clear trend toward higher levels of phagocytosed *slrA* mutants and in addition, counting of attached and phagocytosed pneumococci indicated that the numbers of phagocytosed Δ*slrA* mutants significantly exceeded the numbers of the corresponding wild-type pneumococci (data not shown). In conclusion, the data suggest that SlrA is involved, most likely indirectly, in anti-phagocytic activities.

DISCUSSION

S. pneumoniae expresses two surface-exposed lipoproteins, PpmA and SlrA, which resemble homology with distinct families of PPIases. In this study we have demonstrated PPIase activity of the streptococcal lipoprotein rotamase A, SlrA, and more importantly, we have indicated the importance of SlrA expression for pneumococcal colonization in a mouse infection model and in cell culture infection experiments, respectively.

Colonization and invasive infections of *S. pneumoniae* are associated with the expression of adhesins, or proteins targeting host components. These proteins contribute to colonization or resistance against the innate and adaptive immune defense mechanism of the host. Several surface-exposed proteins such as the choline-binding proteins SpsA (also referred to as CbpA and PspC) (15, 28, 50, 51) and PspA (52, 53), the ABC metal permeases Adc and PsaA (54–56), the plasmin(ogen)-binding proteins (57), and the PavA protein (29, 58) have been shown to be crucial for pneumococcal pathogenesis. However, pneumococcal factors important for the appropriate and efficient folding of translocated or secreted proteins that are involved in pneumococcal pathogenesis have not been addressed yet.

SlrA is homologous with the cyclophilin-type PPIases and enzymatic activity of SlrA toward tetrapeptide substrates was efficiently inhibited by CsA and antiserum generated against SlrA. SlrA showed sequence similarities to the human cyclophilin hCyp18. Nevertheless, substrate specificity was more related to other prokaryotic cyclophilins. Assuming that for the PPIase activity of SlrA a three-dimensional fold similar to human cyclophilin 18 is necessary, three of these extensions are very close together on the surface of the SlrA protein, widening a secondary structure turn element of hCyp18. This region might be of interest with respect to SlrA-specific functional regulation or molecular interaction, as it differs from hCyp18. Only a third of these insertions is present in *Eco*CypB, *San*Cyp18, and *Lp*Cyp18 too (37, 41). The exchange of hCyp18 tryptophan 121 to phenylalanine reduced the affinity toward CsA. Contrarily, the affinity of *Eco*CypB toward CsA was increased by an exchange of the homologous phenylalanine to tryptophan. The corresponding amino acid of SlrA is a tyrosine. This might explain the inhibition constant being higher for SlrA than for hCyp18 but lower

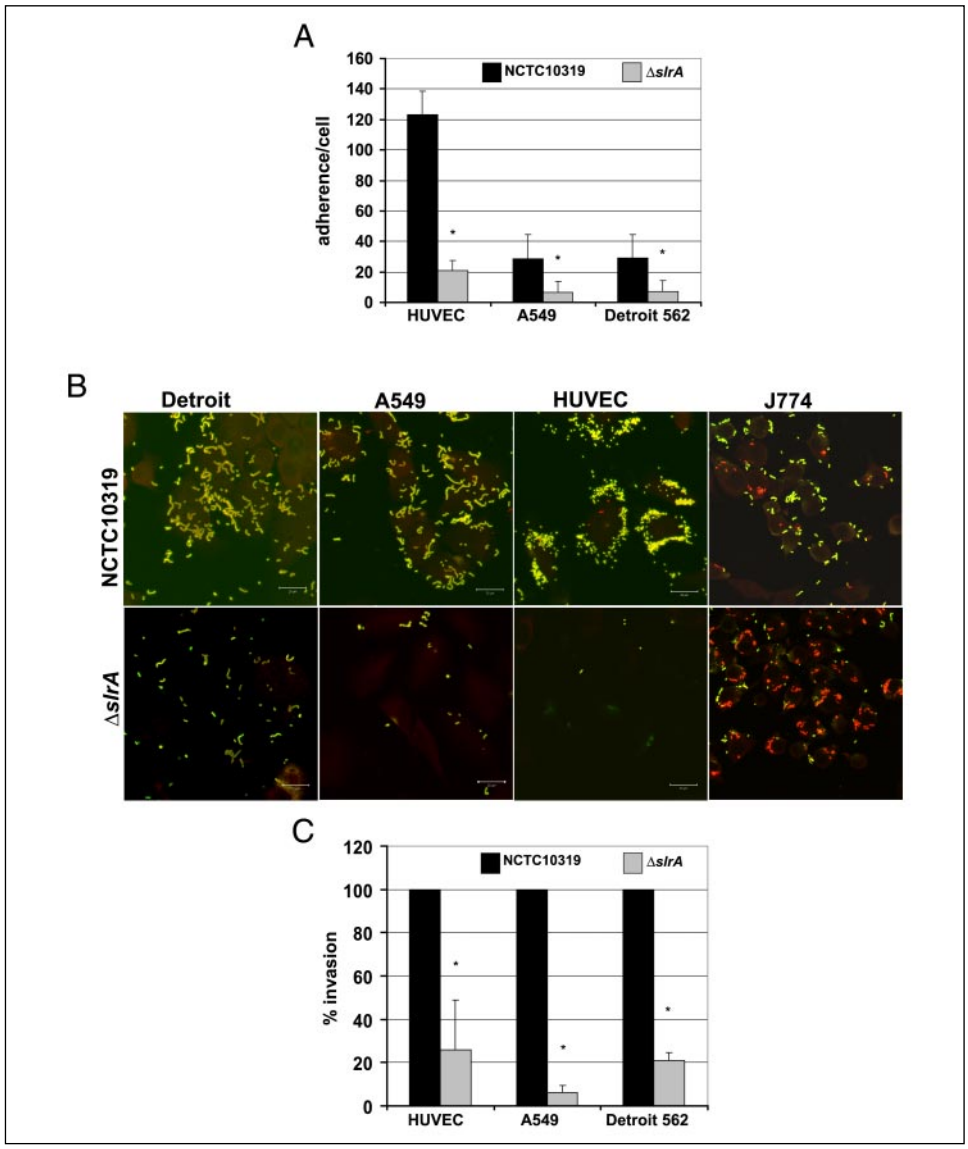

FIGURE 5. **Adherence and internalization of pneumococci and isogenic *slrA* mutants.** Adherence of *S. pneumoniae* strain NCTC10319 (serotype 35A) and its corresponding *slrA* mutant (Δ*slrA*) was numbered by immunofluorescence microscopy after infection of human epithelial cells Detroit 562 and A549, respectively, or endothelial cell line human umbilical vein-derived endothelial cells (*HUVEC*) with a multiplicity of infection of 1:100 (*A*). Double immunofluorescence microscopy of attached (*yellow* (*green plus red*)) and intracellular (*red*) located pneumococci (*B*). Invasive pneumococci is as determined by the antibiotic protection assay (*C*). The number of intracellular surviving NCTC10319 bacteria (serotype 35A) per well was defined as 100% and compared with that of the isogenic *slrA* mutant. Results are expressed as the mean ± S.D. for at least three independent experiments. Statistical significance between wild-type and mutant strains ($p < 0.05$) is depicted by *asterisks*.

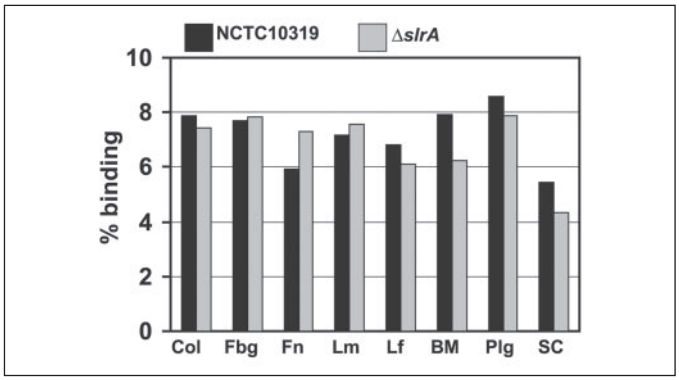

FIGURE 6. **Effect of *slrA* disruption on host protein binding activity of pneumococci.** Binding of fluorescein isothiocyanate-labeled *S. pneumoniae* NCTC10319 (serotype 35A) and its isogenic *slrA* mutant was assayed to 2.5 μg of immobilized host proteins (*Fn*, fibronectin; *Fbg*, fibrinogen; *CollV*, type IV collagen; *Lm*, laminin; *Plg*, plasminogen; *SC*, human secretory component; *Lf*, lactoferrin; or *BM*, Matrigel). The results of the binding studies are expressed as the percentage of the total applied fluorescein isothiocyanate-labeled pneumococci bound to the immobilized proteins. Values (normalized against binding to bovine serum albumin) are from a representative experiment and are the mean of triplicates. Each experiment was performed at least three times with similar results.

than for *Eco*CypB. In contrast to SlrA, the protease-coupled PPIase assay indicated no significant PPIase activity of PpmA, which shares homology with the parvulin family of PPIases. Amino acid sequence alignment over the full-length peptide of PpmA and *E. coli* parvulin 10, representing the prototype of the parvulins, demonstrated 10% sequence similarity and 7% sequence identity. Although the alignment of the putative parvulin region of the much larger PpmA sequence showed that the amino acid sequence similarity is 31% and the identity rises to 23%, PpmA exhibits no PPIase activity. This is consistent with the observation that streptococcal parvulin homologues lack a number of identical amino acids at essential sites of the functional parvulin domain (59).

PPIases form an enzyme class, which accelerate the *cis-trans* isomerization of the peptidyl-prolyl bond in oligopeptides and proteins. Therefore, the absence of PPIase activity in the *slrA* mutant can cause significant changes of the pathogenic potential with respect, *e.g.* to adherence or survival in the host. This hypothesis is supported by the fact that proteins exhibiting PPIase activity such as the Mip of *L. pneumophila* are involved in virulence. Mip-negative mutants were less infective for *Hartmannella vermiformis* and lung epithelial cells (22, 23). Proteins

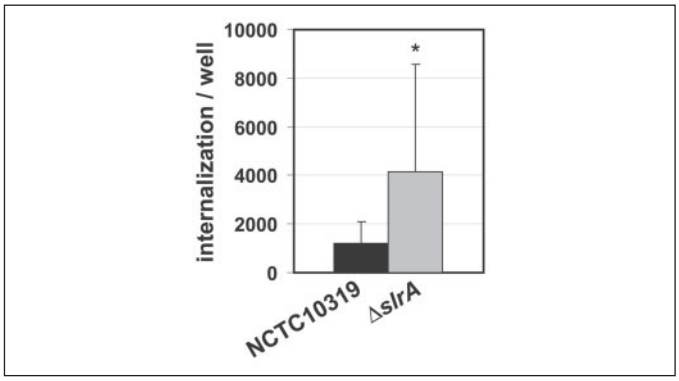

FIGURE 7. **Uptake of *slrA*-deficient pneumococci by J774 macrophages.** Macrophages were infected with wild-type NCTC10319 and isogenic Δ*slrA* mutants, and after 1 h extracellular bacteria were killed by antibiotic treatment. The numbers of internalized wild-type or Δ*slrA* mutants were determined by quantitative plating. The results are expressed as colony-forming units recovered per 24-well (mean ± S.D.) obtained from triplicate experiments. Statistical significance between wild-type and mutant strains ($p < 0.05$) is depicted by an *asterisk*.

with PPIase activity were also identified in other microorganisms such as *Chlamydia trachomatis* (60), *Chlamydia psittaci* (61), *Coxiella burnetti* (62), *Trypanozoma cruzi* (63), and *E. coli* (64). The infectivity of *C. trachomatis* and *T. cruzi* was reduced by the addition of PPIase inhibitors (63, 65). Interestingly, PPIase activity and the structure of Mip are only required for virulence in guinea pig infections, whereas PPIase activity is not required for full virulence of *Legionella* in infections of *Acanthamoeba castellani* (66).

Both pneumococcal lipoproteins have been shown to be immunogenic proteins that are eliciting antibody responses early in life (21), supporting the idea that SlrA and probably also PpmA have important functions in the infection process. Interestingly, the mouse pneumonia model did not show significant differences in the survival times between mice challenged with D39 and isogenic *slrA* mutants. Although under *in vitro* conditions the uptake of a *slrA* mutant by professional phagocytes was significantly enhanced, this effect was not sufficient to increase the survival time in mice challenged with D39Δ*slrA*. It is plausible that the anti-phagocytic effect of the capsular polysaccharide probably covers the deficiency in SlrA during invasive pneumococcal dissemination. However, attenuation of virulence was indicated for pneumococcal mutants lacking the *slrA* gene, because mice challenged with the mutant demonstrated a significantly reduced ability to colonize the nasopharynx and the airways compared with the control strain. Overweg and co-workers (19) have demonstrated an increased survival time, when challenging mice with *ppmA*-deficient mutants. Recent studies using a Δ*slrA*Δ*ppmA* D39 double mutant have demonstrated an additive but not synergistic reduction of virulence for the double mutant compared with the Δ*slrA* and Δ*ppmA* single mutants.[7] This suggests that only two PPIase homologous lipoproteins are not likely to fulfill a homologous function at the surface of *S. pneumoniae*, which is consistent with the observed absence of PPIase activity of PpmA.

The role of SlrA for colonization was confirmed in cell culture infection experiments. Adherence to and internalization in human epithelial and endothelial cells was reduced for pneumococci lacking SlrA. These effects were not because of alterations in the growth rates in cell culture assays or differences in pneumolysin (data not shown). A direct role of SlrA in adherence and invasion was excluded by applying recombinant SlrA protein or anti-SlrA IgG in infection experiments. The presence of rSlrA did not significantly affect the adherence of the wild-type strain and did not complement the adherence of the *slrA* knock-out strain. Adherence was also not changed by preincubation of pneumococci with polyclonal anti-SlrA IgG. A direct function of SlrA as an adhesin was further excluded by applying SlrA-coated microspheres in adherence studies. SlrA-coated microspheres did not bind specifically to epithelial or endothelial cells. Our data further suggested that SlrA did not induce changes on the host cellular surface thereby promoting attachment, which is supported by the fact that anti-SlrA antiserum blocks PPIase activity but did not affect pneumococcal adherence and invasion. The function of SlrA in adherence resembles that of PavA, which affects pneumococcal adherence and virulence by modulating expression or function of important virulence determinants of *S. pneumoniae* (29). However, in contrast to PavA, mutation of *slrA* did not result in complete attenuation of pneumococci.

In addition to directly targeting cellular host receptors such as the polymeric Ig receptor and the platelet-activating factor receptor (15, 67), *S. pneumoniae* binds via surface-located adhesins to a variety of extracellular matrix proteins (44, 46) and serum proteins, such as factor H (68–70), immobilized fibronectin (49, 58), plasmin(ogen) (44, 71), and lactoferrin (46, 52). These pneumococcal adhesins and interactions were shown to be involved in pneumococcal pathogenesis (15, 40, 50, 52, 57). To assess whether SlrA is directly or indirectly involved in pneumococcal binding to SC, reconstituted basement membrane (Matrigel), serum proteins, or proteins of the extracellular matrix, such as fibronectin, type IV collagen, and laminin, respectively, and binding of the *slrA* mutant to these proteins was investigated. The results did not show a significant reduction in binding activity of the SlrA-negative pneumococcal mutant compared with the wild-type, indicating that SlrA has no binding activity for these proteins. In addition, these results indicate that SlrA does not assist in the folding of pneumococcal proteins involved in binding to the above used proteins of the extracellular matrix.

The pneumococcal protein target(s) of SlrA currently remain(s) unknown. Despite the presence of PAPA repeats in choline-binding proteins, which most likely form a proteinase-resistant stalk between the C-terminal choline binding domain and the active peptide domain, the *in vitro* experimental data suggest that there is no interaction between SlrA and choline-binding proteins. Furthermore, because closely related streptococci such as *S. pyogenes*, *Streptococcus mutans*, *Streptococcus agalactiae*, and *Streptococcus suis* possess a SlrA-homologous lipoprotein gene but do not possess choline-binding proteins, it is likely that the role of SlrA is linked to other colonization functions of streptococci.

In conclusion, SlrA represents a novel virulence factor that contributes to pneumococcal pathogenesis in a murine pneumonia infection model, most likely by a non-direct promotion of adherence and invasion of host cells and prevention of pneumococcal phagocytosis. In addition, this study suggests for the first time a link between PPIase-mediated protein folding and pneumococcal virulence and suggests that SlrA may be an interesting therapeutic target for the prevention of host colonization by pneumococci. Because none of the known virulence factors and surface proteins involved in virulence or interaction with host proteins is affected by disruption of the *slrA* gene, the identification of the (virulence) determinants affected by SlrA will be a challenge.

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[7] S. van Selm, personal communication.

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