Reduced Adherence and Host Cell Invasion by Methicillin-Resistant *Staphylococcus aureus* Expressing the Surface Protein Pls

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Pls, the surface protein of methicillin-resistant *Staphylococcus aureus* (MRSA), prevents adhesion of clinical strain 1061 to immobilized fibronectin (Fn) and immunoglobulin G (IgG). Invasion of mammalian cells by *S. aureus* depends on Fn-mediated binding of staphylococcal Fn-binding proteins to host cell β1-integrins. In the present study, we show that, for 10 clinical Pls-positive (Pls+) MRSA strains, adhesion to immobilized Fn, fibrinogen (Fg), IgG, and laminin, as well as binding to soluble Fn and Fg, was less efficient than adhesion and binding associated with 4 Pls-negative (Pls−) MRSA strains. However, binding to soluble IgG was comparable among both types of strains. For 293 cells, Pls+ strains were less invasive than were Pls− strains (median [range], 35% [22%–70%] and 110% [89%–141%], respectively, compared with strain Cowan 1). Disruption of the *pls* gene of strain 1061 increased invasiveness, but it did not affect binding of soluble Fn, Fg, and IgG. Complementation restored the low level of invasiveness, but it did not restore the low level of adhesion to immobilized Fn. In conclusion, the reduced adhesiveness and invasiveness of MRSA appear to generally correlate with expression of Pls.

*Staphylococcus aureus* is a major cause of severe nosocomial and community-acquired infections [1]. Treatment is complicated by the emergence of methicillin-resistant *S. aureus* (MRSA), which may also have acquired multiple-drug resistance. The versatility of this pathogen is reflected by its large number of surface proteins with adhesive and invasive functions. The best characterized of these surface proteins are protein A (SpA), which binds the Fc part of immunoglobulins [2, 3] and von Willebrand factor [4], the fibronectin (Fn)–binding proteins (FnBP) A and B [5–8], the fibrinogen (Fg)–binding proteins ClfA, ClfB, and EfB (Fib) [9–11], the collagen adhesin Cna [12], elastin-binding EbPs [13], and Bbp, which binds bone sialoprotein [14].

Recently, we have constructed a mutant and complemented strain pair for *pls* (gene encoding plasmin-sensitive surface protein) in strain 1061, a Finnish clinical MRSA isolate [15–17]. The *pls* gene is located in the staphylococcal cassette chromosome (SCC)mec, which also contains the mecA gene, coding for a modified penicillin-binding protein 2 (PBP2a or PBP2′) that causes resistance to methicillin (and all other known β-lactams). To date, *pls* has only been found in type I SCCmec element [18, 17]. Pls protein is a homologue of the Sdr (Ser-Asp repeat) family of surface proteins [19], of which ClfA is the best characterized (reviewed in [20]). The Sdr family belongs to the large group of surface-anchored *S. aureus* adhesins (MSCRAMMs [microbial surface components recognizing adhesive matrix molecules]), which are covalently linked to pentaglycine bridges of pep-
Table 1. Bacterial strains used in the present study.

<table>
<thead>
<tr>
<th>Staphylococcus species, strain(s)</th>
<th>Properties</th>
<th>Reference(s) or source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cowan 1</td>
<td>MSSA, pls⁺, isolated from septic arthritis</td>
<td>ATCC 12598</td>
</tr>
<tr>
<td>Newman</td>
<td>MSSA, pls⁺, high-level ClfA expression</td>
<td>ATCC 25904</td>
</tr>
<tr>
<td>1061, 658, 3560, 4210, 5118</td>
<td>MRSA, Finnish clinical isolates, pls⁺</td>
<td>[15]; [16]</td>
</tr>
<tr>
<td>A880510, A900159, A900557, A910071, A920096</td>
<td>MRSA, French clinical isolates, pls⁻</td>
<td>J. Étienne, Centre National de Référence Staphylocoques, Lyon, France; [17]</td>
</tr>
<tr>
<td>1231, 4830, 6207, 6092</td>
<td>MRSA, Finnish clinical isolates, pls⁻</td>
<td>[15]</td>
</tr>
<tr>
<td>1061 pls::Tc⁺</td>
<td>Pls⁺</td>
<td>[17]</td>
</tr>
<tr>
<td>1061 pls::Tc⁺ (pPLS4)</td>
<td>Pls⁺</td>
<td>[17]</td>
</tr>
<tr>
<td>Undesignated isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSSA (n = 20)</td>
<td>MSSA, isolated from patients with osteomyelitis (4) or endocarditis (9) and from the nares of healthy carriers (7)</td>
<td>J. Schrenzel, University Hospitals of Geneva, Geneva, Switzerland</td>
</tr>
<tr>
<td>MRSA (n = 8)</td>
<td>Isolated, randomly, from clinical specimens in 1998</td>
<td>B.S., at University Hospitals of Geneva, Geneva, Switzerland</td>
</tr>
<tr>
<td>S. carnosus, TM300</td>
<td>No expression of known adhesins</td>
<td>[30]</td>
</tr>
<tr>
<td>S. epidermidis, N860348, N860187, Hall, 190</td>
<td>Isolated from blood cultures of patients with endocarditis</td>
<td>J. Étienne, Centre National de Référence Staphylocoques, Lyon, France; [31]</td>
</tr>
</tbody>
</table>

NOTE. MRSA, methicillin-resistant S. aureus; MSSA, methicillin-susceptible S. aureus; pls⁺, positive for the pls gene; pls⁻, negative for the pls gene; Pls⁺, positive for the Pls protein; Pls⁻, negative for the Pls protein.

* Does not possess any known genes related to S. aureus adhesins.

tidoglycan through a consensus sequence (LPXTG) by sortase [21]. Disruption of pls increases the poor adhesion of strain 1061 to rabbit IgG and human Fn [17].

Previously, we and others have elucidated the molecular mechanism of cellular invasion by S. aureus [22–26]. Invasion proceeds as an actin polymerization-dependent zipper-type mechanism and is dependent on FnBPs and host cell αβ, integrins, which are linked by Fn. Extracellular adhesion protein recently has been shown to partially compensate for the absence of functional FnBPs as invasins [27].

The aim of the present study was to examine (1) whether the presence of pls/Pls inversely correlates with adhesive and invasive properties in MRSA isolates and (2) whether disruption of the pls gene restores the invasive phenotype in an isogenic background to levels observed in Pls-negative (Pls⁻) strains.

MATERIALS AND METHODS

Reagents, enzymes, and antibodies. Recombinant lysostaphin (Ambicin L) was supplied by Applied Microbiology, and human serum albumin (HSA) was supplied by Behring. Fn, which was isolated from plasma samples from humans, and laminin, which was isolated from Engelbreth-Holm-Swarm mouse tumor (entactin free), were obtained from BD Biosciences. Human Fg was obtained from either Kabi or Kordia Life Sciences, and bovine serum albumin (BSA) was obtained from Sigma. Rabbit IgG was purified from antiserum raised against Escherichia coli Dr fimbriae [28]. Smal was obtained from Roche, and the lambda ladder (#N0340S) was obtained from New England Biolabs.

Alkaline phosphatase–conjugated rabbit anti–mouse IgG and swine anti–rabbit IgG were obtained from Dako A/S. Monoclonal anti–protein A antibody was obtained from Sigma. Polyclonal anti–ClfA antibody, raised against amino acid residues 40–559 of domain A in ClfA, was a gift from Tim Foster (Trinity College, Dublin, Ireland). Monoclonal antibodies against Pls purified from strain 1061 were produced, as described elsewhere [29], with the modification that RPMI was substituted for Dulbecco’s modified Eagle medium (DMEM).

Bacterial strains and cultures. S. aureus Newman and Cowan 1 strains and S. carnosus strain TM300 were used as reference isolates. The bacterial strains that were used are listed in table 1. Twenty clinical methicillin-susceptible S. aureus (MSSA) isolates, 8 MRSA isolates, and 4 S. epidermidis isolates were used in an initial study of invasiveness among staphylococcal isolates. Fourteen clinical MRSA isolates that were different from the aforementioned isolates were characterized in more detail and were used for studies of adhesion and invasion. The clinical MSSA isolates were obtained during 1990–1995 and were provided by Jacques Schrenzel (Division of Infectious Diseases, University Hospitals of Geneva, Geneva, Switzerland). For adhesion and protein-binding assays, bacteria were grown to stationary phase in tryptic soy broth (TSB) at 37°C with agitation. TSB was supplemented with chloramphenicol (5 μg/mL) for strain 1061 pls::Tc⁺ (pPLS4). The bacteria were washed with PBS and were quantified using a Petroff-Hausser counting
chamber (Hausser Scientific Partnership). Preparation of bacteria for the invasion assays is described below.

**Iodination of proteins.** Rabbit IgG, human Fn, and human Fg were iodinated using the iodogen method [32], as described in detail elsewhere [33], with an incubation time of 1 min.

**Pulsed-field gel electrophoresis (PFGE) and Southern hybridization.** For PFGE, *S. aureus* DNA was digested with Smal and was separated on 1% agarose gels, as described elsewhere [34]. Gels were run in 0.5 × Tris borate EDTA buffer (45 mmol/L Tris, 45 mmol/L boric acid, 1 mmol/L EDTA, pH 8), by use of a clamped homogeneous electric field (CHEF)–DR II electrophoresis cell (BioRad), under the following conditions: 14°C, a switch time of 5–50 s, a 120-degree angle, and 6 V/cm.

For Southern analysis, staphylococcal DNA was digested with *Pst* I and *Stu* I [17]; digestion was followed by detection of *pls* and *clfA* on a positively charged nylon membrane, by use of the digoxigenin (DIG) nonradioactive nucleic acid labeling and detection system (Roche). Probes for *pls* and *clfA* were prepared by amplification of the *A* regions of genes from strains 1061 and Newman, respectively, by use of primers described elsewhere [17, 35].

**Detection of MRSA surface proteins.** Surface proteins were solubilized by incubation of *S. aureus* cells with lysostaphin in the presence of raffinose, essentially as described elsewhere [36]. For detection of Pls and SpA, surface proteins were prepared from exponential- or stationary-phase cultures, and, for detection of Fn-binding proteins and ClfA, surface proteins were prepared from exponential-phase cultures grown in TSB. For Western analysis, proteins were separated by SDS-8% polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes with a pore size of 0.2 μm (Protran). Membranes were blocked with 2% BSA (wt/vol) in PBS. Pls and SpA were visualized by probing the membranes with predetermined dilutions of monoclonal mouse anti-Pls and anti-SpA antibodies, followed by treatment with rabbit anti–mouse IgG conjugated to alkaline phosphatase and alkaline phosphatase substrate. For the 8 undesignated MRSA strains used in the initial study of invasiveness, Pls was visualized using sheep antiserum to affinity-purified Pls and peroxidase-conjugated MIG5, as detailed elsewhere [17]. ClfA was detected with rabbit anti–ClfA antibody and alkaline phosphatase–conjugated swine anti–rabbit IgG. For detection of proteins binding Fn, ligand overlay assays were used instead of antibody-based visualization. Blocked nitrocellulose membranes were incubated with 125I-Fn in PBS containing 1% of BSA, for 16 h at 22°C under mild agitation, and they were then washed with PBS and exposed to an X-Omat x-ray film (Kodak). The surface protein analyses were performed only once.

**Adhesion of staphylococci to immobilized host proteins.** Bacterial adhesion to protein-coated glass slides was tested essentially as described elsewhere [37]. In brief, glass slides were coated with 40 μL of human Fn (14.6 μg/mL), human Fg (10 μg/mL; treated with Sepharose-denatured type I collagen to remove possible trace amounts of Fn), rabbit IgG (10 μg/mL), mouse laminin (51 μg/mL), or BSA (25 μg/mL) in PBS for 16 h at 22°C. Thereafter, 40-μL suspensions that contained 5 × 10^6, 10^7, 5 × 10^7, and 10^8 bacteria/mL in PBS were incubated on BSA-saturated slides for 2 h at 22°C. Adherent methylene blue–stained bacteria were visualized using a microscope that was equipped with a charge-coupled device camera, and the images were digitized using the National Institutes of Health Image 1.55 program, as detailed elsewhere [38]. The numbers of bacteria in 24 randomly chosen microscopic fields of 1.6 × 10^4 μm^2 were determined. The adhesion assays were performed twice. Results from 1 representative experiment are shown.

**Binding of soluble proteins.** Five nanograms of 125I-labeled IgG or Fg (specific activities, 2.0 × 10^6 or 1.7 × 10^6 cpm/μg, respectively) was incubated with 10^8 or 5 × 10^8 bacteria, and 50 ng of 125I-Fn (specific activity, 1.0 × 10^5 cpm/μg) was incubated with 4 × 10^8 or 2 × 10^8 bacteria in PBS containing 0.1% BSA (wt/vol), for 1 h at 22°C with gentle agitation. The specificity of binding was controlled by incubating the bacteria with a 1000-fold molar excess of the corresponding unlabeled protein (competitor) for 30 min before the labeled protein was added. The cells were washed with 1 mL of 0.1% BSA-PBS, and the radioactivity bound to the cells was measured using a gamma counter. The binding assay was performed twice for IgG and Fg and once for Fn. Results from a representative experiment are shown.

**Preparation of fluorescein-5-isothiocyanate (FITC)–labeled bacteria.** For FITC-labeling, bacteria were prepared as described elsewhere [22]. In brief, bacterial cultures were grown overnight in 5 mL of Müller-Hinton broth (Mast) at 37°C without agitation. Strain 1061 *plc*:Tc^R* (pPLS4) was grown in the presence of 10 μg/mL chloramphenicol. Bacteria were washed with 0.9% NaCl, were fixed in 1% formaldehyde in PBS for ≥1 h, and then were washed again. The bacteria subsequently were treated with 3 mL of 0.5-mol/L NaHCO₃ buffer (pH ~9.5) supplemented with 100 μg/mL FITC (isomer I [Molecular Probes]; solubilized in 150 μL of DMSO) for 1 h at 37°C. Finally, bacteria were resuspended in PBS that contained 1% HSA (wt/vol), and they were used within 24 h after preparation. Suspensions were normalized for an optical density of 1 at 540 nm, after gentle sonication in a water bath.

**Cell culture.** All media components were obtained from Gibco-BRL. Adenovirus type 5 DNA-transformed primary human embryonic kidney cells (293 cells) were obtained from the ATCC (#CRL-1573), were maintained in DMEM/Nut mix F-12 (containing Glutamax I, a stable glutamine dipeptide), were supplemented with 10% fetal calf serum (FCS), 50 IU/mL penicillin, and 50 μg/mL streptomycin, and were split 1:4 twice.
weekly, by trypsinization. Before the cells were used in the experiments, they had been passaged for a maximum of 35 times after freezing.

Flow cytometric invasion assay. MRSA strains were assayed for cellular invasion, in a blinded fashion, by use of a flow cytometric invasion assay described elsewhere [22, 23], with minor modifications. A fresh bacterial culture was used for each experiment. In brief, the day before the assay, 293 cells were plated in 24-well plates at 0.4 × 10⁶ cells per well. Cells were washed with DMEM/Nut mix F-12; this was followed by the addition of 0.5 mL of invasion medium (DMEM/Nut mix F-12 supplemented with 1% HSA and 10 mmol/L HEPES, pH 7.4). The cells were cooled on ice, and 50-μL suspensions of fixed, FITC-labeled bacteria were added, resulting in an estimated MOI of ~25:1. Cells were preincubated for 1 h at 4°C, to allow sedimentation of bacteria; subsequently, the conditions were changed to 37°C for 3 h, to allow for invasion. Finally, cells were harvested, were treated with 20 μmol/L monensin (for 10 min at ambient temperature) to neutralize fluorescence quenching, and were analyzed by flow cytometry, after propidium iodide (5 μg/mL) exclusion, as described elsewhere [22]. Results were normalized according to the mean fluorescence intensity of the respective bacterial preparation, as determined by flow cytometry. The results were expressed as the mean ± SEM of ≥3 independent experiments performed in duplicates.

Statistical analysis. Student’s 2-tailed t test was used for comparison of Pls-positive (Pls⁺) and Pls⁻ strains. If variances between the groups were not equal, the t test probabilities were calculated using the Cochran and Cox approximation. P < .05 and P < .001 were considered to be statistically significant and highly statistically significant, respectively.

RESULTS

PFGE analysis and characterization of strains. All isolates were confirmed, by automated phenotyping, to be S. aureus and, as appropriate, MRSA (identification and susceptibility testing for oxacillin; Vitek II [BioMérieux]). In addition, all strains were phenotypically compared on sheep blood agar (comparisons of colony size, pigmentation, and hemolysis were performed). As opposed to MSSA isolates, MRSA isolates with SCCmec types I–III [39], but not type IV [40], tend to have a higher probability of being clonally related. To exclude clonal identity between the strains used in the present study, genomic PFGE analysis was performed. Only isolates that were obtained from different patients and that showed ≥1 band difference were included (figure 1). A few of the strains were closely related to each other, showing a difference of 1–3 bands. The majority, however, had larger differences that suggested either more distant or no relatedness. Although these criteria only allow the exclusion of identical isolates, not related isolates, the results presented are based on a selection of strains with rather diverse genetic backgrounds.

Prevalence of the pls gene and its expression. Four of the Finnish MRSA strains were pls negative, as determined by Southern hybridization with a pls region A probe. All other MRSA strains were pls positive (figure 2 and table 1). Pls was expressed by all strains that carried the pls gene (Pls⁺ strains), except for the pls disruption mutant strain 1061 pls::Tc, which served as a negative control, as determined by Western blot analysis (figure 3). The band adjacent to the 45-kDa marker is likely to be protein A that binds IgG nonspecifically.

Expression of adhesins. Expression of a panel of surface adhesins likely to be relevant for the present study was analyzed to ensure that the different behavior in the adhesion and invasion assays was not caused by a lack of expression of surface adhesins. Proteins binding to Fn were expressed by almost all the strains, as judged by the ability of bands in lysostaphin digests blotted on nitrocellulose to bind radiolabeled Fn. Fn was mainly bound to 2 high-molecular-weight bands, but it was also bound to several bands with a lower molecular weight. In addition to FnBP’s, the 40-kDa extracellular matrix protein–binding protein (Emp) and the 65–75-kDa extracellular adherence protein (Eap) of S. aureus have also been shown to bind Fn [41, 42]. The presence of ClfA and SpA was studied using specific antibodies. Most Pls⁻ isolates expressed ClfA more strongly than did the Pls⁺ isolates studied; some of the Pls⁻ strains either showed very low (barely detectable, by West-
tern blot analysis) or no ClfA expression. The clfA gene, however, was present in all strains studied, as detected by Southern blot analysis (not shown). Some strains expressed protein A at very low levels. However, there did not seem to be a difference in the expression of SpA between Pls+ and Pls strains. Expression of surface adhesins by the strains is shown in table 2.

**Adhesion to immobilized host proteins.** The clinical MRSA strains that expressed Pls showed lower adhesion to immobilized Fn than did MRSA strains that were devoid of Pls and the MSSA reference strain Cowan 1 (P < .001 between Pls+ MRSA and Pls− strains including Cowan 1). Only one of the Pls− strains was able to adhere to immobilized Fg, whereas all Pls− MRSA and strain Cowan 1 adhered effectively (P < .001 between Pls+ and Pls− strains). Pls+ MRSA strains were unable to adhere to immobilized IgG or laminin. Two Pls+ MRSA strains adhered to IgG, and 3 adhered to laminin effectively. (IgG adhesion: P < .05, for the comparison of strains expressing protein A [table 2] in groups of Pls+ and Pls− strains, for 10⁸ bacteria/mL, and P < .1 [statistical trend], for 5 × 10⁷ bacteria/mL; laminin adhesion: P < .1 [statistical trend], for 10⁸ bacteria/mL, and P < .05, for 5 × 10⁷ bacteria/mL). None of the strains adhered to BSA that was used as a negative control (data not shown). Disruption of pls in strain 1061 resulted in a markedly increased adherence to Fn and IgG and a moderately increased adherence to Fg, whereas adherence to laminin was not affected. However, complementation of pls failed to decrease adhesion to immobilized Fn to low wild-type (wt) levels (figure 4).

**Binding of soluble host proteins.** Binding of soluble Fn was the most prominent difference between the 2 groups. On average, Pls+ strains bound soluble Fn and Fg more effectively than did Pls− strains (P < .001 [for Fn binding] and P < .1 [for Fg binding] [statistical trend], in a comparison of Pls+ MRSA and Pls− strains, including strains Cowan 1 and Newman). Binding of IgG, in contrast to adherence to immobilized IgG, was effective for most of the strains, regardless of whether they were Pls+ or Pls− (no significant difference between the groups). However, there was not much difference between strains 1061 and 1061 pls::TcR, with regard to binding any of the 3 proteins (figure 5).

**Invasion of host cells.** In our initial experiments, when the invasiveness of clinical *S. aureus* isolates was analyzed, we found that a group of randomly obtained MRSA isolates was only weakly invasive, compared with clinical MSSA isolates (median invasiveness, relative to reference strain Cowan 1: 21% and 103%, respectively; median invasiveness of clinical *S. epidermidis* strains, 10%) (figure 6). Because invasion of host cells by *S. aureus* is dependent on Fn binding, and because the presence of pls reduces adherence to immobilized Fn, whereas disruption of pls increases adherence to Fn, we tested a possible role for pls in reducing invasiveness. When analyzed retroactively for the presence of pls, almost all of these clinical MRSA isolates were pls positive. However, this was not the case for 2 isolates, which were pls negative and were only weakly invasive.

To clearly determine the effect of pls on invasiveness, we tested, in a blinded fashion, the collection of MRSA strains characterized in the present study, with regard to invasiveness. After the presence of pls was decoded, 2 groups of isolates could be distinguished with regard to invasiveness: MRSA strains expressing Pls displayed a markedly reduced cellular invasiveness, compared with Pls− MRSA strains and the MSSA strain Cowan 1 (figure 7A). The 4 Pls+ MRSA strains were as invasive as was the Cowan 1 strain (median invasiveness, 110%; range, 89%–141%), whereas the 10 Pls− MRSA strains were less invasive (median invasiveness, 35%; range, 22%–70%) (P < .05, for the comparison of Pls+ and Pls− MRSA isolates). This effect could be reproduced using genetically defined mutants. Disruption of pls led to increased invasiveness, which could be restored at least to wt (i.e., lower) levels following complementation of the mutation (figure 7B). The mean cellular invasiveness (± SEM) of isolate 1061 was between 26% ± 2% and 49% ± 5% for the wt, 79% ± 11% for the pls mutant, and 26% ± 11% for the complemented mutant. Taken together, pls− MRSA strains appear to be as invasive as MSSA isolates, whereas pls+ MRSA isolates are markedly less invasive than MSSA isolates.

**DISCUSSION**

We have previously shown that Pls is responsible for the poor adhesion of MRSA strain 1061 to immobilized Fn and IgG [17]. In addition, in the present study, we show that the in-
Reduces Adhesion and Invasion

Figure 3. Western blot analysis of Staphylococcus aureus cell wall extracts, by use of monoclonal anti-Pls antibodies. Molecular size markers are shown. Pls is partly seen as a smaller cleavage product (size, 230 and 175 kDa in strain 1061) [16, 17]. Size variation is the result of variation in repeat numbers in different strains [17]. Pls+, positive for the pls gene; Pls−, negative for the pls− gene.

The adhesion and invasion properties of MRSA strains devoid of Pls very much resemble those of MSSA strains, and reduced adhesiveness and invasiveness appear to be exclusively associated with Pls, which is found on type I SCCmec. Overall, reduced cellular invasiveness appears to be the exception in clinical S. aureus isolates, restricted to pls-positive MRSA.

Table 2. Expression of surface adhesins.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pls</th>
<th>Fn-binding proteins</th>
<th>ClfA</th>
<th>Protein A</th>
</tr>
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<tbody>
<tr>
<td>1061</td>
<td>+</td>
<td>+</td>
<td>/−</td>
<td>++</td>
</tr>
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<td>+</td>
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<td>1061 pls::Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>−</td>
<td>+</td>
<td>/−</td>
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<td>1061 pls::Tc&lt;sup&gt;+&lt;/sup&gt; (pPLS4)</td>
<td>+</td>
<td>+</td>
<td>/−</td>
<td>++</td>
</tr>
<tr>
<td>Cowan 1 (MSSA)</td>
<td>−</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Newman (MSSA)</td>
<td>−</td>
<td>ND</td>
<td>++</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE.** Levels of expression were quantified visually and are given semiquantitatively, independently for each adhesin. Fn, fibronectin; MSSA, methicillin-susceptible S. aureus; ND, not determined; ++, high expression; +, expression; /−, very low expression; −, no expression detected.

vasiveness of strain 1061 is impaired in the presence of pls. Furthermore, the degree of adhesiveness and invasiveness of various MRSA isolates seems to generally correlate with expression of Pls.

For MRSA strains that express Pls, poor binding to Fg (figures 4 and 5) can be explained, at least in part, by reduced expression of ClfA, compared with MRSA strains not producing Pls. Lack of clumping-factor activity has been previously reported for MRSA [43]. Even if all the strains evaluated in the present study had the clfA gene, it was poorly expressed by most Pls+ MRSA strains (table 2). In contrast, protein A and FnBPs were equally prevalent and were expressed in similar levels in both groups of MRSA. The few exceptions with regard to expression of SpA correlate well with reduced adhesion (table 2 and figures 4 and 5). The importance of FnBPs for S. aureus is emphasized by the high prevalence of FnBPs in clinical isolates; in 2 studies, all of 25 strains [44] and all but 1 of 30 [45] strains tested had ≥1 fnb gene, as detected by Southern blot analysis of total DNA or PCR-amplified regions of fnb<sub>A</sub> and fnb<sub>B</sub>. In yet another study, all of the 163 MSSA strains that were analyzed by Southern dot blot analysis had ≥1 of the fnb genes [46].

The molecular biology of staphylococcal laminin binding is not well characterized. Pls+ MRSA strains adhered very poorly to laminin, compared with Pls− MRSA strains (figure 4). Because several distinct functions are affected, it is likely that Pls has a general influence on adhesins that contain the LPXTG motif. This was, however, difficult to demonstrate in the present study, because of the lack of expression of some adhesins on MRSA strains. This hypothesis is supported by experiments that demonstrate that introduction of a mec element that contained pls caused MSSA strains to become not only methicillin resistant but, also, nonadhesive to immobilized Fn and Fg, without reduction in expression of adhesins [47].
protein (Aap) of *S. epidermidis*, which is essential for biofilm formation on surfaces by *S. epidermidis* [48]. It has been shown that addition of purified Pls to a suspension of MRSA leads to bacterial aggregation; this finding suggests a role for Pls in promoting interactions between bacterial cells [49]. However, it is not known whether expression of Pls is also associated with biofilm formation in *S. aureus*. SasG, an *S. aureus* homologue of Aap and Pls, recently has been described [50], and it appears to mediate binding to human desquamated nasal epithelial cells [51]. Pls appears to have 2 opposing functions. It is antiadhesive and anti-invasive when plasma or matrix proteins are involved, but it may be adhesive in some other contexts (e.g., through binding to lipids [49] or to desquamated keratinocytes [51]).

Complementation of the *pls*:Tc<sup>h</sup> mutation by plasmid-encoded *pls* was unable to restore the adhesiveness to immobilized Fn to low wt levels (figure 4). The reason for this is currently unclear. The addition of soluble Pls increases bacterial adhesion to different immobilized host ligands, such as Fn, Fg, IgG, and mucin [49]. The overexpression of Pls might have a similar

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**Figure 4.** Adhesion of bacterial cells to glass slides coated with human fibronectin (Fn) (A) or fibrinogen (Fg) (B), rabbit IgG (C), or mouse laminin (Lam) (D). None of the strains adhered to bovine serum albumin used as a negative control (not shown). Results with 2 bacterial concentrations are shown. Results are missing for laminin with bacteria/mL for strains A900159 and A920096. Results are means of 1 representative experiment (for details of the statistical analysis, see the “Adhesion to immobilized host proteins” subsection in Results). bact., Bacteria; Pls +, positive for the Pls protein; Pls −, negative for the Pls protein.
Figure 5. Binding of radiolabeled, soluble fibronectin (Fn) (A), fibrinogen (Fg) (B), and IgG (C) to Staphylococcus aureus. Results with 2 bacterial concentrations and with a 1000-fold molar excess of unlabeled protein (competitor) from 1 representative experiment are shown (for details of the statistical analysis, see the “Binding of soluble host proteins” subsection in Results). bact., Bacteria; Pls⁺, positive for the Pls protein; Pls⁻/H11002, negative for the Pls protein.
Figure 6. Invasiveness of clinical Staphylococcus aureus and Staphylococcus epidermidis (S. epi) isolates. Twenty methicillin-susceptible S. aureus (MSSA) isolates that originated from patients with osteomyelitis or endocarditis and from the nares of healthy carriers and 4 S. epidermidis isolates that originated from patients with endocarditis were compared with 8 methicillin-resistant S. aureus (MRSA) isolates randomly obtained from different specimens. Strains were assayed exactly as described elsewhere [22]. Results are the means ± SEM of 3–4 independent experiments run in duplicates, expressed as relative invasiveness, compared with strain Cowan 1. Solid lines, median invasiveness of each group; dotted lines, mean invasiveness of each group. The pls gene and its expression were detected as described in Materials and Methods. Six MRSA strains were pls positive; 5 of these strains were also Pls positive. One strain was Pls positive in spite of having a negative result by Southern blot analysis. One strain was pls negative and Pls negative (open circle).

effect, and, thus, it could compensate for reduced adherence. However, in this case, it would be hard to explain why invasiveness was restored to a low wt level by pls complementation of the disruption mutant (figure 7), as expected.

The present investigation revealed differences in the interaction of Pls+ MRSA with soluble and solid-phase host proteins. Although adhesion of these strains to immobilized IgG was almost totally prevented (figure 4), binding of soluble IgG to the strains was not affected (figure 5). In addition, this difference was clearly visible with wt strain 1061, its isogenic pls disruption mutant, and the complemented mutant. This effect could be explained by steric hindrance (i.e., a better accessibility of soluble IgG, compared with immobilized IgG, for SpA on the bacterial surface), and it may reflect the mechanism by which Pls prevents the function of SpA.

Figure 7. Invasion of 293 cells by defined methicillin-resistant Staphylococcus aureus strains. Total internalized bacteria was measured by flow cytometry with fixed bacteria. Results are the means ± SEM of 3 independent experiments run in duplicates, and they are expressed as relative invasiveness, compared with strain Cowan 1 (for details of the statistical analysis, see Results). Pls+, Pls positive; Pls−, Pls negative.

FnBP-mediated binding of bacteria to host cells certainly is the main route of efficient S. aureus invasion. Consequently, a reduced Fn-binding capacity of strains that express Pls also leads to an impaired ability to invade eukaryotic cells. The role of FnBPs as virulence factors is still somewhat controversial in in vivo models of infection. A low-Fn-binding transposon mutant of S. aureus showed reduced adherence to traumatized heart valves in a rat model of endocarditis [52]. By contrast, another study that used the same model found no difference between the virulence of strain 8325-4 and that of its isogenic FnBP-negative mutant [53]. Expression of FnBPA in Lactococcus lactis, however, was sufficient to cause high infectivity in rat traumatic endocarditis [54]. On the other hand, in a rat model of pneumonia, an FnBP deletion mutant of strain 8325-4 showed increased growth and acute lung injury, compared with the wt strain, at 24 h after infection. However, in vitro internalization of wt bacteria by alveolar epithelial cells was still FnBP dependent [55]. These data are not easy to interpret, because strain 8325-4 has a regulatory defect (rsbU) [56], expresses FnBPs at a low level [57], and is only weakly invasive in vitro [22]. Nevertheless, if this holds true, decreased S. aureus
adhesion to Fn and cellular invasion might even, in some circumstances, contribute to pathogenesis of S. aureus.

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References


