Protein A-Mediated Multicellular Behavior in *Staphylococcus aureus*[∇]

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The capacity of *Staphylococcus aureus* to form biofilms on host tissues and implanted medical devices is one of the major virulence traits underlying persistent and chronic infections. The matrix in which *S. aureus* cells are encased in a biofilm often consists of the polysaccharide intercellular adhesin (PIA) or poly-*N*-acetyl glucosamine (PNAG). However, surface proteins capable of promoting biofilm development in the absence of PIA/PNAG exopolysaccharide have been described. Here, we used two-dimensional nano-liquid chromatography and mass spectrometry to investigate the composition of a proteinaceous biofilm matrix and identified protein A (*spa*) as an essential component of the biofilm; protein A induced bacterial aggregation in liquid medium and biofilm formation under standing and flow conditions. Exogenous addition of synthetic protein A or supernatants containing secreted protein A to growth media induced biofilm development, indicating that protein A can promote biofilm development without being covalently anchored to the cell wall. Protein A-mediated biofilm formation was completely inhibited in a dose-dependent manner by addition of serum, purified immunoglobulin G, or anti-protein A-specific antibodies. A murine model of subcutaneous catheter infection unveiled a significant role for protein A in the development of biofilm-associated infections, as the amount of protein A-deficient bacteria recovered from the catheter was significantly lower than that of wild-type bacteria when both strains were used to coinfect the implanted medical device. Our results suggest a novel role for protein A complementary to its known capacity to interact with multiple immunologically important eukaryotic receptors.

Staphylococcus aureus is a gram-positive bacterium that lives as part of the normal microflora on the skin and mucous membranes of humans and animals. If *S. aureus* passes through the epithelial barrier and reaches internal organs, it can cause a variety of diseases, ranging from minor skin infections, such as furuncles or boils, to severe infections, such as bacteremia, pneumonia, osteomyelitis, or endocarditis. Despite the progress with antibiotics in the treatment of bacterial infections over the last 2 decades, the number of infections due to *S. aureus* has increased (11, 30). The infection rate has been correlated with an increase in the use of prosthetic and indwelling devices in modern medical practices (24, 26). *S. aureus*, as well as other coagulase-negative staphylococci, displays a strong capacity to irreversibly attach to the surface of implanted medical devices and forms multilayered communities of bacteria, known as biofilms, that grow embedded in a self-produced extracellular matrix (23). The biofilm formation process occurs in two steps: first, bacterial cells irreversibly attach to a surface, and second, they interact with each other and accumulate in multilayered cell clusters embedded in a self-produced extracellular matrix. Primary attachment is mediated by physico-chemical cell surface properties as well as specific factors that mediate the attachment to the host-derived extra-

cellular matrix components that rapidly coat the biomaterial following insertion into the patient. Numerous proteins from the MSCRAMMs family (microbial surface components recognizing adhesive matrix molecules) are involved in the first step of *S. aureus* biofilm formation, such as clumping factors ClfA (37) and ClfB (41) and fibrinogen and fibronectin binding proteins (FnBPA and FnBPB) (25, 31). Once bacteria accumulate in multilayered cell clusters, most have no direct contact with the surface, and thus cell-to-cell interactions become essential for biofilm development and maintenance. An extracellular polysaccharide intercellular adhesin (PIA, or PNAG), produced by *icaADBC* operon-encoded enzymes, is currently the best-characterized element mediating intercellular interactions in vitro (8, 23, 34, 35, 38). Alternatively, a number of surface proteins can replace PIA/PNAG exopolysaccharide in promoting intercellular adhesion and biofilm development, including the surface protein Bap (9). All the tested staphylococcal isolates harboring the *bap* gene were shown to be strong biofilm producers, and inactivation of the *icaADBC* operon in *bap*-positive strains had no effect on in vitro biofilm formation (57). Remarkably, proteins homologous to Bap are involved in the biofilm formation process in diverse bacterial species (33). A second surface protein, SasG, as well as its homologous protein in *Staphylococcus epidermidis*, Aap, also mediates intercellular interactions and biofilm development in the absence of the *ica* operon (7, 51). More recently, two independent laboratories have shown that fibronectin binding proteins A and B (FnBPA and FnBPB) induce biofilm development of

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TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Reference
S. aureus strains		
ISP479r	ISP479c *rsbU* restored	56
ISP479r Δ*arlRS*	ISP479r Δ*arlRS* operon	56
ISP479r *agr*	ISP479r *agr*::*tet*	56
ISP479r *agr* Δ*spa*	ISP479r *agr*::*tet* Δ*spa*	This study
ISP479r *agr* Δ*arlRS*	ISP479r *agr*::*tet* Δ*arlRS* operon	56
ISP479r *agr* Δ*arlRS srtA*	ISP479r *agr*::*tet* Δ*arlRS srtA*::*ermC*	This study
ISP479r *agr* Δ*arlRS* Δ*spa*	ISP479r *agr*::*tet* Δ*arlRS* Δ*spa*	This study
ISP479r *agr* Δ*arlRS* Δ*sdrD*	ISP479r *agr*::*tet* Δ*arlRS* Δ*sdrD*	This study
ISP479r *agr* pCN40	ISP479r *agr* transformed with pCN40 plasmid	This study
ISP479r *agr* Δ*arlRS* pCN40	ISP479r *agr* Δ*arlRS* transformed with pCN40 plasmid	This study
ISP479r *agr* Δ*arlRS* Δ*spa* pCN40	ISP479r *agr* Δ*arlRS* Δ*spa* transformed with pCN40 plasmid	This study
ISP479r *agr* Δ*arlRS* Δ*spa* pCN40*spa*	ISP479r *agr* Δ*arlRS* Δ*spa* complemented with *spa* gene	This study
Newman		12
Newman *srtA*	Newman *srtA*::*ermC*	36
Newman *spa*	Newman *spa*::*Km*	44
Newman pCN40	Newman transformed with pCN40 plasmid	This study
Newman pCN40*spa*	Newman complemented with *spa* gene	This study
Newman *agr*	Newman *agr*::*tet*	This study
Newman *agr spa*	Newman *agr*::*tet spa*::*Km*	This study
12313	Clinical strain isolate	56
Lactococcus lactis strains		
MG1363 pKS80	Plasmid-cured *L. lactis* strain complemented with pSK80 plasmid	44
MG1363 pKS80*spa*	Plasmid-cured *L. lactis* strain complemented with *spa* gene	44
Escherichia coli XL1-Blue	Used for cloning assays	
Plasmids		
pMAD	*E. coli-S. aureus* shuttle vector with *bgaB* gene encoding a β-galactosidase; Ap^r/Er^r	1
pMAD *spa*AD	pMAD plasmid containing the mutant allele for deletion of *spa* gene	This study
pMAD *sdrD*AD	pMAD plasmid containing the mutant allele for deletion of *sdrD* gene	This study
pCN40	*E. coli-S. aureus* shuttle vector with constitutive promoter P$_{blaZ}$	5
pCN40*spa*	pCN40 plasmid containing *spa* gene	This study
pCN40*spa*ΔLPXTG	pCN40 plasmid containing *spa* gene without charged tail and hydrophobic domain	This study

clinical isolates of *S. aureus* (45, 55). Finally, there is growing evidence that extracellular DNA, despite not being sufficient to replace PIA/PNAG exopolysaccharide, is an important *S. aureus* biofilm matrix component (50).

During the course of a systematic mutagenesis study of the 17 two-component systems of *S. aureus* that aimed to identify biofilm-negative regulators, we found that *S. aureus agr arlRS* double mutants developed an alternative, *ica*-independent biofilm in a chemically defined medium, Hussain-Hastings-White (HHW) medium (56). This study focused on the identification of the proteinaceous compound responsible for the biofilm developed by *S. aureus agr arlRS* mutants. Here, we show that *S. aureus* protein A is responsible for the aggregative phenotype and capacity for biofilm formation displayed by this strain. Furthermore, overproduction of protein A in wild-type *S. aureus* strains or addition of soluble protein A to bacterial growth medium induced aggregation and biofilm development, suggesting that protein A does not need to be covalently linked to the cell wall to promote multicellular behavior. Moreover, deletion of the *spa* gene significantly decreased the capacity of *S. aureus* to colonize subcutaneously implanted catheters. Our findings support a novel role for protein A in promoting multicellular behavior and suggest that protein A-mediated biofilm development may have a critical function during the infection process of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The most relevant bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* XL1-Blue cells were grown in Luria-Bertani broth or on Luria-Bertani agar (Pronadisa, Madrid, Spain) with appropriate antibiotics. Staphylococcal strains were cultured using different media: trypticase soy agar (TSA), trypticase soy broth supplemented with glucose (0.25%, wt/vol) (TSBg), and chemically defined HHW modified (HHWm) medium. *Lactococcus lactis* strains were incubated in M17 medium (Pronadisa, Madrid, Spain). Media were supplemented with appropriate antibiotics at the following concentrations: erythromycin (Er), 20 μg ml^{-1}, 1.5 μg ml^{-1}, or 10 μg ml^{-1}; ampicillin (Am), 100 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; tetracycline (Tet), 10 μg ml^{-1}. When required, TSA was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Bioline, London, United Kingdom).

DNA manipulations. DNA plasmids were isolated from *E. coli* strains using the Qiagen plasmid mini prep kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. Plasmids were transformed into staphylococci by electroporation, using a previously described protocol (9). Restriction enzymes were purchased from Takara Shuzo Co. Ltd. or New England Biolabs and used according to the manufacturers' instructions. Oligonucleotides were obtained from Thermo (Electron Corporation). The *srtA* gene was inactivated in *S. aureus* ISP479r *agr* by transferring *srtA*::*ermC* from *S. aureus* Newman *srtA* (36) by phage transduction using φ85 (42).

Allelic exchange of chromosomal genes. To construct the deleted strains, we amplified by PCR two fragments of approximately 800 bp that flanked the left side (oligonucleotides A and B) and the right side (oligonucleotides C and D) of the sequence targeted for deletion (Table 2). The two obtained fragments were cloned in the pGEM-T Easy vector (Promega). Oligonucleotides B and C carry the same restriction site at the 3′ and 5′ ends, respectively, so that it is possible

TABLE 2. Oligonucleotides used in the study

Name	Sequence
spa-A	5'-<u>GAATTC</u>CAATTCTAGCTATTATCACTT-3'
spa-B	5'-<u>TCTAGA</u>AATTAATACCCCCTGTATGTA-3'
spa-C	5'-<u>TCTAGA</u>AAACAAACAATACACAACGAT-3'
spa-D	5'-<u>GGATCC</u>TTAAAATGGAAAAGTGCAGG-3'
spa-E	5'-GATGATGTATACAATGTATTTC-3'
spa-F	5'-TGCGTCTCGATTTAATTGG-3'
spa-G	5'-<u>GAATTC</u>CACTTAAAACCATTTCCGAA-3'
spa-H	5'-<u>GGATCC</u>ATCGTTGTGTATTGTTTGTTT-3'
pCN40spa-1	5'-<u>GGATCC</u>AAAGTTGTAAAACTTACATTTAAA-3'
pCN40spa-2	5'-<u>GAATTC</u>ATCGTTGTGTATTGTTTGTTT-3'
pCN40spa-3	5'-<u>GAATTC</u>TTATGATAATCCACCAAATACAG-3'
sdrD-A	5'-<u>GGATCC</u>GTTACAGAAATGTAATTGTCTATG-3'
sdrD-B	5'-AGTTGCTGCATATCAACTTTATC-3'
sdrD-C	5'-CAGCTTATACCAGGTCCGTG-3'
sdrD-D	5'-<u>CCATGG</u>TGTGTTGAATCATTTTCTGTTGTCG-3'
sdrD-E	5'-GATTAACTTAACCAGGTCCATG-3'
sdrD-F	5'-CTTTAGCTTCTGGTTGTGAATC−3'
arlRS-E	5'-<u>GGATCC</u>TTGATTGATTGACGTCTCAGTCAT-3'
arlRS-F	5'-<u>AAGCTT</u>GTGAAATGTTTAAACTACGG-3'
spa-Fw	5'-GCAAACGGCACTACTGCTGA-3'
spa-Rv	5'-CACCAGTTTCTGGTAATGCTTGAG-3'
gyr-Fw	5'-TTATGGTGCTGGGCAAATACA-3'
gyr-Rv	5'-CACCATGTAAACCACCAGATA-3'

to fuse fragments AB and CD by ligation, creating the AD fragment. Besides, oligonucleotides A and D carry restriction sites, so that it is possible to fuse the AD fragment to the shuttle plasmid pMAD previously digested with the corresponding enzymes. The resulting plasmids were transformed into *S. aureus* by electroporation. pMAD contains a temperature-sensitive origin of replication and an erythromycin resistance gene (1). Homologous recombination experiments were performed as previously described (60). Erythromycin-sensitive white colonies, which no longer contained the pMAD plasmid, were tested by PCR using oligonucleotides E and F to confirm the gene replacement.

Complementation studies. The *spa* gene was amplified with thermophilic DNA polymerase (Certamp long amplification kit; Biotools, Spain) from *S. aureus* strain ISP479r with primers pCN40spa-1 and pCN40spa-2 (Table 2). pCN40 is a staphyloccus-*E. coli* shuttle vector that harbors the constitutive PblaZ promoter (43). The PCR product was cloned into pCN40 (pCN40spa) so that it was expressed under the PblaZ promoter. This plasmid and the vector control were introduced in the different strains by phage transduction using φ85 (42). The *spa* gene lacking the carboxy-terminal region was amplified from strain ISP479r by PCR with primers pCN40spa-1 and pCN40spa-3 and it was cloned into pCN40 to create the pCN40spa ΔLPXTG plasmid. This plasmid was introduced in ISP479r *agr* ΔarlRS Δspa by phage transduction using φ85.

Surface-associated protein preparation. Surface protein preparations were obtained from *S. aureus* cells under iso-osmotic conditions. Bacterial cells were recovered from 5-ml overnight cultures by centrifugation, washed twice in 1 ml of phosphate-buffered saline (PBS), and resuspended in 100 μl of iso-osmotic digestion buffer (PBS containing 26% [wt/vol] raffinose). After addition of 3 μl of a 1-mg/ml solution of lysostaphin (Sigma), the preparations were incubated with shaking at 37°C for 2 h. Protoplasts were sedimented by centrifugation at 8,000 × *g* for 30 min with slow deceleration, and the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or stored at −20°C.

Biofilm formation assays. A biofilm formation assay in microtiter wells was performed as described previously (28). Briefly, 5 μl of a culture of *S. aureus* grown overnight in HHWm or TSBg at 37°C was inoculated into the wells of microtiter plates (Iwaki) containing 195 μl of HHWm or TSBg (final dilution of the culture, 1:40). After 24 h of incubation at 37°C, the microplates were washed three times with 200 μl of H₂O, dried in an inverted position, and stained with 100 μl of 0.25% crystal violet for 2 to 3 min at room temperature. Then, the microplates were rinsed again three times with H₂O and dried, the dye was subsequently dissolved in 200 μl of ethanol-acetone (80:20), and the absorbance was measured at 595 nm. For the biofilm formation inhibition assay we used a nonspecific rabbit serum, purified immunoglobulin G (IgG [I5006]; Sigma), and anti-protein A-specific antiserum (P3775; Sigma) at concentrations ranging from 500 μg/ml to 3.6 μg/ml. Then, a biofilm formation assay was performed as described above. To analyze biofilm formation under flow conditions, we used 60-ml microfermentors (Pasteur Institute; (www.pasteur.fr/recherche/unites/Ggb /biofilmfermenter.html) with a continuous flow of 40 ml of chemically defined medium h⁻¹ and constant aeration with sterile pressed air. Submerged Pyrex

slides served as the growth substratum. Bacteria from an overnight culture grown at 37°C with aeration in chemically defined media were diluted to an optical density (OD) at 600 nm of 1. For the inoculations, the Pyrex slides were introduced in the bacterial solution for 2 min and placed back in the microfermentors. The microfermentors were cultivated for 24 h at 37°C. Biofilm development was recorded with a Nikon Coolpix 950 digital camera. To quantify the 24-h biofilm, bacteria that adhered to the surfaces of the Pyrex slides were resuspended in 10 ml of chemically defined medium. The OD of the suspension was measured at 650 nm. Biofilm formation assays in the presence of supernatants were performed as follows. Supernatants from ISP479r *agr* ΔarlRS (supernatant 1), ISP479r *agr* ΔarlRS Δspa complemented with pCN40spa (supernatant 2), ISP479r *agr* ΔarlRS Δspa (supernatant 3), and ISP479r *agr* ΔarlRS Δspa complemented with a truncated version of protein A that lacks the LPXTG domain (supernatant 4) were obtained after centrifugation of an overnight culture at 2,800 rpm for 20 min. Filtered supernatants were added 1:1 (vol/vol) to ISP479r *agr* ΔarlRS Δspa cultures. Soluble commercial protein A (P3838; Sigma) was added at a concentration of 10 μg/ml. Then, the biofilm formation assay was performed as previously described.

Cell aggregation assay. Liquid cultures of *S. aureus* ISP479r *agr* ΔarlRS, ISP479r *agr* ΔarlRS Δspa, Newman pCN40, Newman pCN40spa, and *Lactococcus lactis* MG1363 pKS80 and MG1363 pKS80spa were separately grown overnight in HHWm, TSBg, or M17. Cultures were adjusted to an OD₆₀₀ of 2.5. Cultures of 3 ml were incubated in 5-ml standing tubes at room temperature, and the OD₆₀₀ of the upper part of the culture was measured throughout time. Assays were performed in triplicate.

Electrophoresis and Western blot analysis. Electrophoretic separation of cell wall-associated protein preparations was carried out by SDS-PAGE. The acrylamide concentration was 12% in the resolving gel and 5% in the stacking gel. For Western blotting, proteins were transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare) by electroblotting. The buffer used for transfer was 50 mM Tris (pH 8.3)–380 mM glycine–0.1% SDS–20% methanol. The blocking solution contained PBS, 5% milk powder, and 0.1% Tween 20. Probing was carried out only using a secondary antibody taking into account the property of protein A to bind the Fc region of the Igs. Nitrocellulose membranes were washed with PBS-Tween and then incubated for 1 h at room temperature with a 1:5,000 dilution of a peroxidase-conjugated AffiniPure rabbit anti-sheep IgG (Jackson InmunoResearch Laboratories, Inc.). The reaction was developed using the ECL Western blotting analysis system (GE Healthcare). Images were obtained with a Chemi-Doc apparatus (Bio-Rad).

Preparation of *S. aureus* strains for the two-dimensional nano-liquid chromatography and ion trap mass spectrometric (2DnLC-MS/MS) analysis. Bacterial strains were grown overnight at 37°C in HHW growth medium. A 200-ml aliquot of fresh medium was then inoculated with a 1:10 dilution of the overnight culture and grown until an OD₆₅₀ of 1 was reached. Bacteria were spun down by centrifugation (14,000 rpm, 10 min, 4°C) and washed twice with a 1/10 volume of the original culture in PBS. The bacteria were fixed for 1 h at 4°C in 20 ml of a solution containing 3% paraformaldehyde and then washed twice with ultrapure H₂O. Fixed bacteria were resuspended in a 1/10 volume of the original culture of ammonium bicarbonate, 50 mM, and modified trypsin (sequencing grade; Promega, Madison, WI) was added at a final amount of 1 μg per sample. Digestion with trypsin was performed under shaking conditions for 1 h at 37°C. Digested peptides were separated from the bacterial cells by centrifugation (300,000 × *g*, 20 min, 30°C). The supernatant containing the peptide mixture was lyophilized and kept at −20°C.

2DnLC-MS/MS analysis. The tryptic peptide mixtures (4 μg each) were injected onto a strong cationic exchange micro-precolumn (500 μm inner diameter [ID] by 15 mm; BioX-SCX; LC Packings, Amsterdam, The Netherlands) with a flow rate of 30 μl/min as a first-dimension separation. Peptides were eluted from the column as fractions by injecting three salt steps of increasing concentrations of ammonium acetate (10, 100, and 2,000 mM). Each of the three fractions together with the nonretained fraction was on-line injected onto a C₁₈ reversed-phase microcolumn (300 μm ID by 5 mm; PepMap; LC Packings) to remove salts, and the peptides were analyzed in a continuous acetonitrile gradient consisting of 0 to 50% B in 45 min and 50 to 90% B in 1 min (B is 95% acetonitrile, 0.5% acetic acid in water) on a C₁₈ reversed-phase self-packing nano-column (100 μm ID by 15 cm; Discovery BIO Wide pore; Supelco, Bellefonte, PA). A flow rate of 300 nl/min was used to elute peptides from the reversed-phase nanocolumn to a PicoTip emitter nano-spray needle (New Objective, Woburn, MA) for real-time ionization and peptide fragmentation on an Esquire HCT ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). Every 1 s, the instrument cycled through acquisition of a full-scan mass spectrum and one MS/MS spectrum. A 4-Da window (precursor *m/z* ± 2), an MS/MS fragmentation amplitude of 0.80 V, and a dynamic exclusion time of 0.30 min were used for

peptide fragmentation. 2DnLC was automatically performed on an advanced microcolumn switching device (Switchos; LC Packings) coupled to an autosampler (Famos; LC Packings) and a nano-gradient generator (Ultimate nano-HPLC; LC Packings). The software Hystar 2.3 was used to control the whole analytical process.

Database analysis. MS/MS spectra were batch processed by using DataAnalysis 5.1 SR1 and MS BioTools 2.0 software packages and searched against the *S. aureus* protein databases using Mascot software (Matrix Science, London, United Kingdom).

Real-time quantitative PCR. Total *S. aureus* RNA was prepared using the Fast RNA-Blue kit (Bio101) according to the manufacturer's instructions. Two micrograms of each RNA was subjected in triplicate to DNase I (Gibco-BRL) treatment for 30 min at 37°C. The enzyme was inactivated at 65°C in the presence of EDTA. To verify the absence of genomic DNA in every sample, the RNA triplicates were reverse transcribed in the presence and absence of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). All preparations were purified using CentriSep spin columns (Princeton Separations). A 1/20 fraction of each reaction mixture was used for real-time quantitative PCR using a Light-Cycler and the SYBR Green PCR master mix (Applied Biosystems). The *spa* transcripts were amplified using primers *spa*-FW and *spa*-RV (Table 2). The *gyr*B transcripts that are constitutively expressed were amplified as endogenous controls using primers *gyr*-FW and *gyr*-RV (63) (Table 2). To monitor the specificity, final PCR products were analyzed by melting curves and electrophoresis. Only samples with no *gyr*B amplification of the minus reverse transcriptase aliquot were considered in the study. The amount of *spa* transcript was expressed as the difference relative to the control gene ($2^{-\Delta C_t}$, where ΔC_t represents the difference in threshold cycle between the target and control genes).

Immunofluorescence microscopy. Immunofluorescence microscopy analysis was performed as previously described (61). Overnight cultures of the different strains were diluted to an OD_{650} of 1 in HHW and 10 μl was loaded onto 0.1% poly-(L-lysine)-treated immunofluorescence microscope slides. Slides were washed three times with PBS between each step of this protocol. Cells were fixed with 3% paraformaldehyde for 10 min before quenching with 50 mM NH_4Cl in PBS for 3 min. Slides were then saturated for 15 min with 0.5% bovine serum albumin in PBS before being incubated with a 1:200 dilution of a secondary anti-mouse IgG (whole molecule) antibody produced in rabbit (Sigma) conjugated to tetramethyl rhodamine isothiocyanate (TRITC). Finally, the slides were mounted with Vectashield HardSet mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories) and observed under an epifluorescence microscope with rhodamine and DAPI filters.

Experimental infection. A mouse foreign body infection model was used to determine the role of protein A in the pathogenesis of *S. aureus*. A total of 60 adult male mice (Swiss-Albino) were used. A 1-cm segment of intravenous catheter (24 gauge; B. Braun) was aseptically implanted into the subcutaneous interscapular space. Each group of mice was coinoculated with 10^5 CFU of both *S. aureus* Newman and the *spa* mutant strain (*spa*::Km). The animals were euthanized by cervical dislocation on days 3 or 7 postinfection. The catheter was aseptically removed, placed in a sterile tube containing 1 ml of TSBg, sonicated, and vortexed at high speed for 3 min. The CFU/catheter was determined by plating bacteria in either TSA or TSA-Km (50 μg/ml). An extra group of animals was inoculated with vehicle (PBS) and served as a negative control. The experiment was repeated twice.

Statistical analysis. A two-tailed Student's test with Welch's correction was used to assess significant differences in bacterial recovery within groups in experimental infections. Differences were considered statistically significant when *P* was <0.05. The GraphPad 5.0 statistical package was used (GraphPad Software, San Diego, CA).

RESULTS

Non-gel analysis of the *S. aureus* cell wall proteome. To identify the protein component responsible for the PIA/PNAG-independent biofilm development in *agr arlRS* mutants, we first generated a mutant of the sortase A gene (*srtA*) in ISP479r *agr* Δ*arlRS* strain. Sortase A was selected because it catalyzes the cell wall anchoring of the LPXTG family of surface proteins. Biofilm formation assays revealed that deletion of *srtA* completely inhibited the biofilm phenotype displayed by the *agr* Δ*arlRS* mutant, suggesting that an LPXTG protein was essential for the biofilm-forming capacity of these strains (data
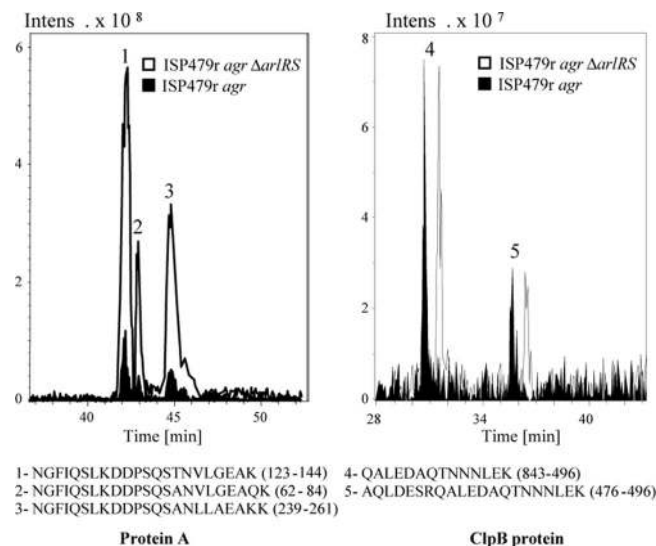

1- NGFIQSLKDDPSQSTNVLGEAK (123-144)　　　4- QALEDAQTNNNLEK (843-496)
2- NGFIQSLKDDPSQSANVLGEAQK (62- 84)　　　5- AQLDESRQALEDAQTNNNLEK (476-496)
3- NGFIQSLKDDPSQSANLLAEAKK (239-261)

Protein A　　　　　　　　　　　　　　　**ClpB protein**

FIG. 1. EICs of protein A and ClpB in *S. aureus* ISP479r *agr* and ISP479r *agr* Δ*arlRS* strains. On the left is the EIC from ions at *m/z* 783.38 (ion 1), 816.07 (ion 2), and 825.4 (ion 3) Da, corresponding to protein A-derived tryptic, triply charged peptides spanning sequences shown below. Ionic intensity from the three detected signals was increased fivefold in the Δ*arlRS* sample. On the right is the EIC for ions at *m/z* 794.37 (ion 4) and 796.37 (ion 5) Da, corresponding to doubly and triply charged tryptic peptides, respectively, from the ClpB protein, which is expressed at the same levels in both samples. Retention times for the ISP479r *agr* Δ*arlRS* sample have been shifted 1 minute to avoid chromatogram overlapping.

not shown). The cell wall proteomes of ISP479r *agr* and its corresponding isogenic *arlRS* mutant were then compared by 2DnLC-MS/MS. Fixed bacterial cells were digested with trypsin as described in Materials and Methods to yield a peptide mixture that was fractionated and analyzed by 2DnLC coupled to electrospray ionization and mass spectrometry (4). Two LPXTG proteins, protein A and SdrD, were detected in the cell wall proteome of the ISP479r *agr* Δ*arlRS* mutant (data not shown). In contrast to SdrD, protein A was also detected in the cell proteome of the ISP479r *agr* strain. To confirm that the levels of protein A were higher on the surface of ISP479r *agr* Δ*arlRS* compared to the wild-type ISP479r *agr* strain, the intensities of extracted ion chromatograms (EIC) were compared. As shown in Fig. 1, the intensity of the ions corresponding to the trypsin-digested peptides from protein A was fivefold higher in the Δ*arlRS* sample, confirming that protein A is present at higher levels in the *arlRS* mutant compared to the wild-type strain. In contrast, and as a control, analysis of the EIC corresponding to the tryptic peptides from the ClpB protein showed that the intensity of the signals was almost identical in both strains, indicating that the amount of ClpB on the surface of both bacteria is similar. Overall, these results demonstrate that the LPXTG proteins, protein A and SdrD, were present at higher levels on the cell surface of the *S. aureus agr* Δ*arlRS* double mutant than in the *agr*-deficient strain.

Deletion of the *spa* gene causes a decrease in the biofilm development promoted by the *arlRS* mutation. We then asked whether either of the two LPXTG proteins whose levels are augmented in double *agr arlRS* mutant strains was responsible for the biofilm formation phenotype. We deleted the *spa* and

sdrD genes in the ISP479r *agr* Δ*arlRS* strain. Biofilm assays in polystyrene petri dishes revealed that only deletion of the *spa* gene inhibited the biofilm formation capacity, whereas deletion of *sdrD* had no effect (Fig. 2A). This result indicated that protein A was responsible for the development of biofilm in *agr* Δ*arlRS* strains. To verify this finding, complementation studies were performed. When pCN40*spa* was introduced into ISP479r *agr* Δ*arlRS* Δ*spa*, the biofilm phenotype was completely restored, confirming that inactivation of *spa* was responsible for the biofilm negative phenotype of this strain (Fig. 2B). Immunoblotting assays demonstrated that the levels of protein A were elevated in the ISP479r *agr* Δ*arlRS* strain compared to the corresponding single mutants and wild-type strain (Fig. 2C). To investigate whether this increase of protein A expression observed in the *agr arlRS* mutant was due to augmented expression of the *spa* gene, we employed real-time quantitative PCR. In agreement with previous results (16), the levels of *spa* gene expression were not affected by *arlRS* deletion (Fig. 2D). Taken together, these results indicated that protein A is an essential component of the biofilm produced by *agr* Δ*arlRS* strains and that these strains accumulate higher levels of protein A on their cell surface, promoting biofilm development in vitro.

Protein A mediates biofilm development in the absence of a functional *agr* system. Recent studies have suggested a role for the *agr* system in *S. aureus* biofilm development, as *agr* mutants exhibit a high propensity to form biofilms, and cells dispersing from a biofilm have been observed to display an active *agr* system (3, 64). Thus, we wondered whether protein A might be responsible for the increased biofilm development displayed by *agr* mutants. As shown in Fig. 2A, disruption of the *spa* gene in the *agr* mutant completely inhibited biofilm development, suggesting that increased accumulation of protein A in the absence of an active *agr* system is responsible at least in some strains for the tendency of *agr* mutants to develop stronger biofilms.

Protein A induces aggregation and biofilm formation under flow cell conditions. To further investigate whether the increased levels of protein A in *agr* Δ*arlRS* strains were able to induce other phenotypes associated with multicellular behavior, we monitored cell-to-cell interactions in an aggregation assay and biofilm formation capacity in a continuous flow culture on glass spatulas submerged in microfermentors (19). In contrast to protein A-deficient bacteria that remained in suspension, *S. aureus agr* Δ*arlRS* strongly aggregated and rapidly sedimented on the bottom of the tube (Fig. 3A). Accordingly, visualization of both macroscopic biofilm development in microfermentors and biofilm cell density on the removable glass spatulas inside the microfermentors showed that *S. aureus agr* Δ*arlRS* formed a robust biofilm after 24 h of incubation, in contrast to its corresponding *spa* mutant (Fig. 3B). Together, these results indicate that protein A promotes cell-to-cell interactions and biofilm formation under flow conditions.

Expression of protein A leads to increased aggregation and biofilm formation in wild-type *S. aureus* strains. We then asked whether deletion or overexpression of protein A might affect biofilm formation in wild-type *S. aureus* strains. We first inactivated *spa* in *S. aureus* Newman strain, which does not synthesize PIA/PNAG exopolysaccharide and displays a slight or null capacity to form a biofilm in microtiter plates in
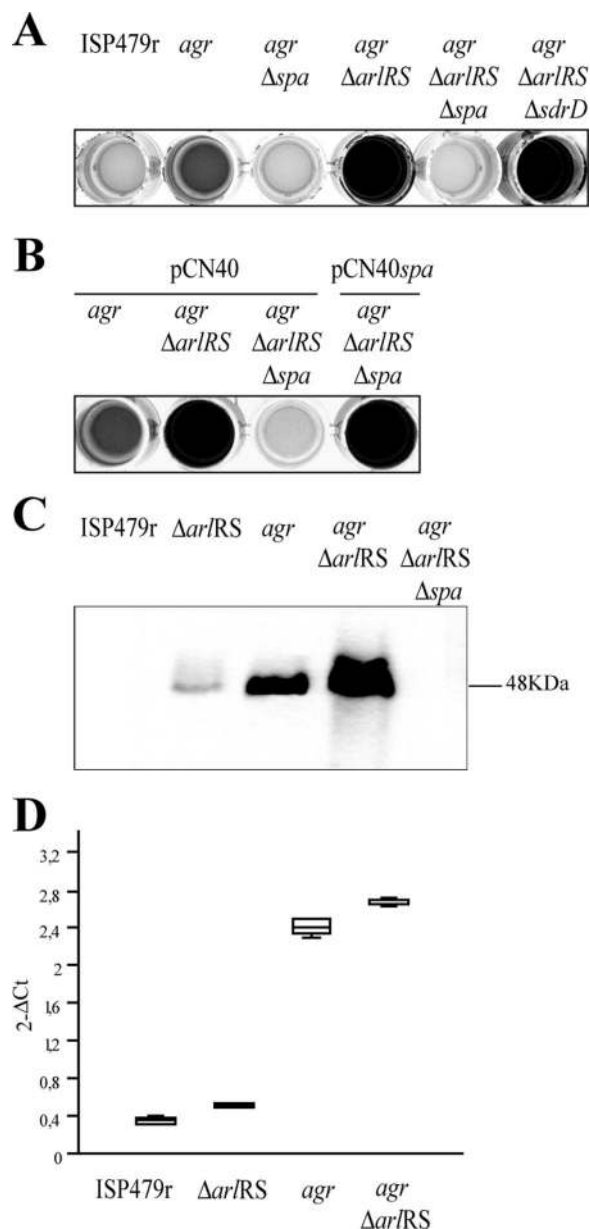

FIG. 2. Biofilm formation phenotype in polystyrene microtiter dishes in HHWm medium after 24 h of incubation. (A) *S. aureus* ISP479r, *S. aureus* ISP479r *agr*, *S. aureus* ISP479r *agr* Δ*spa*, ISP479r *agr* Δ*arlRS*, ISP479r *agr* Δ*arlRS* Δ*spa*, and ISP479r *agr* Δ*arlRS* Δ*sdrD* strains. (B) *S. aureus* ISP479r *agr*, ISP479r *agr* Δ*arlRS*, and ISP479r *agr* Δ*arlRS* Δ*spa* strains with the pCN40 control plasmid and *S. aureus* ISP479r *agr* Δ*arlRS* Δ*spa* strain complemented with the pCN40*spa* plasmid carrying the *spa* gene under the P_{blaZ} promoter. HHWm was supplemented with 10 μg/ml of erythromycin as needed. (C) Western blot analysis for protein A expression of *S. aureus* ISP479r, ISP479r Δ*arlRS*, ISP479r *agr*, ISP479r *agr* Δ*arlRS*, and ISP479r *agr* Δ*arlRS* Δ*spa* strains. The blot was probed with a 1:5,000 dilution of a peroxidase-conjugated AffiniPure rabbit anti-sheep IgG. (D) Real-time quantification of *spa* gene expression in *S. aureus* strains ISP479r, ISP479r Δ*arlRS*, ISP479r *agr*, and ISP479r *agr* Δ*arlRS* at mid-log exponential phase (OD_{650}, 0.6).

HHWm. As shown in Fig. 4, deletion of *spa* reduced the capacity of the strain to produce a biofilm in microfermentors, but it did not cause any noticeable effect on the capacity of the strain to produce a biofilm in microtiter plates. In agreement
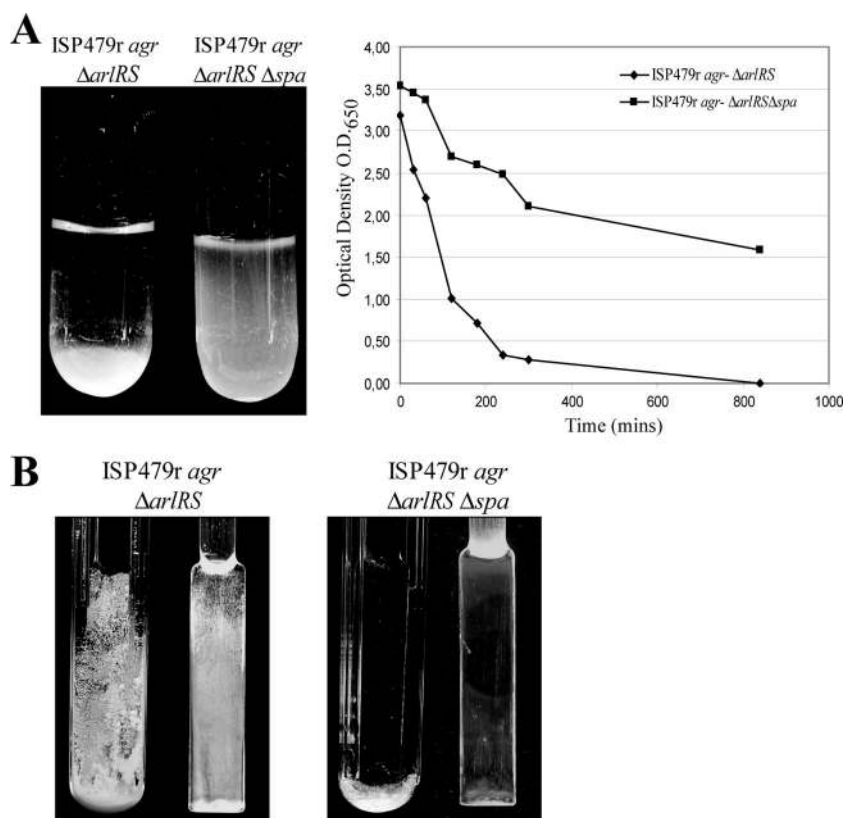
FIG. 3. (A) In vitro aggregation assay of *S. aureus* ISP479r *agr* Δ*arlRS* and ISP479r *agr* Δ*arlRS* Δ*spa* strains. On the left side the aggregation phenotype observed in glass tubes is shown, and on the right side the quantification of the optical density at 650 nm at different times is displayed. Results of a representative experiment are shown. (B) Biofilm development of *S. aureus* ISP479r *agr* Δ*arlRS* and ISP479r *agr* Δ*arlRS* Δ*spa* strains grown in microfermentors under continuous flow of HHWm for 24 h at 37°C. The microfermentor (left) and the glass slides where bacteria form the biofilm (right) are shown.

with previous results, mutation of *agr* bestowed the capacity to produce a biofilm in microtiter plate assay. Such increased biofilm formation capacity was completely inhibited when the *spa* gene was inactivated in the *S. aureus* Newman *agr* strain, indicating that protein A was required for increased biofilm development in the absence of *agr*.

Then, we expressed protein A in *S. aureus* strain Newman from the multicopy plasmid pCN40. The results revealed that overexpression of protein A elicited aggregation in liquid culture and biofilm development in both polystyrene microtiter plates and microfermentors under flow conditions (Fig. 5A and B). To generalize the effects of protein A in multicellular behavior, we heterologously expressed protein A in *Lactococcus lactis* (Fig. 5C) and observed an increased aggregation phenotype indicative of bacterial clumping. Overall, these results demonstrate that production of protein A is sufficient to mediate biofilm development in wild-type *S. aureus* strains and cell-to-cell interactions in both wild-type *S. aureus* and *L. lactis* strains.

Antibodies inhibit protein A-induced biofilm formation. Protein A directly reacts with the Fc portion of immunoglobulins (14). The IgG-binding regions of protein A consist of four or five domains, depending on the *S. aureus* strain (32). Based on this property, we reasoned that nonimmune binding of IgG to protein A could sterically interfere with the capacity of the protein to induce cell-cell interactions and biofilm formation. To test this hypothesis, we performed biofilm formation assays of *S. aureus* ISP479r *agr* Δ*arlRS*, *S. aureus* Newman pCN40*spa*, and *S. aureus* 12313 strains under the presence of increasing concentrations of a rabbit nonspecific serum, purified IgG, or polyclonal antibodies raised against protein A. The *S. aureus* 12313 strain was included as a control in the study, as its biofilm formation capacity depends on the production of the PIA/PNAG exopolysaccharide. We found that biofilm formation capacity of the ISP479r *agr* Δ*arlRS* and Newman pCN40*spa* strains was completely inhibited in a dose-dependent manner by nonspecific rabbit serum, purified IgG, and anti-protein A antibodies (Fig. 6). However, similar concentrations of antibodies did not affect the biofilm formation capacity of the clinical *S. aureus* 12313 strain. Taken together, these results indicate that interaction of antibodies with protein A sterically block the capacity of protein A to mediate biofilm development; this finding provides further evidence of the requirement for protein A in the biofilm development process.

Extracellular protein A induces biofilm formation. Protein A belongs to the LPXTG family of proteins characterized by its covalent linkage to the cell wall peptidoglycan. However, most *S. aureus* strains secrete significant levels of protein A (15 to 30%) into the cell culture (13). This prompted us to investigate
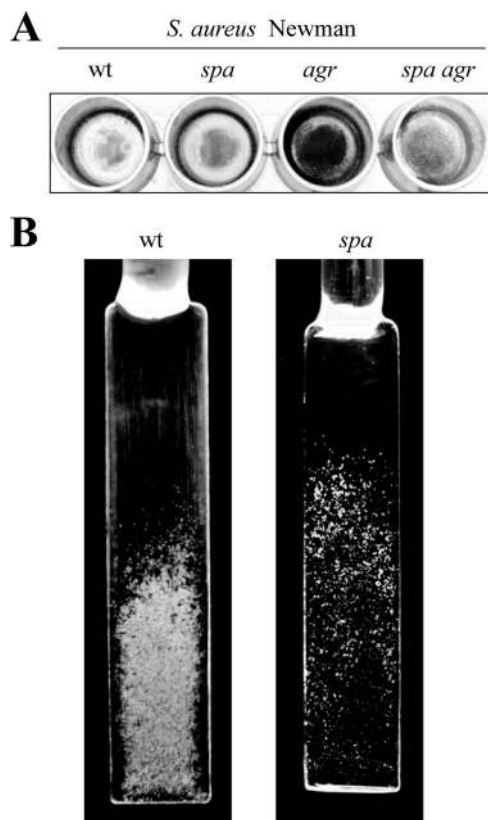
FIG. 4. (A) Biofilm formation phenotype in polystyrene microtiter dishes with *S. aureus* Newman wild-type strain and its corresponding *spa*, *agr*, and *spa agr* mutant strains. (B) Biofilm development of *S. aureus* Newman wild-type strain and its corresponding *spa* mutant strain grown in continuous flow of HHWm for 24 h at 37°C. The glass slides where bacteria formed the biofilm are shown.
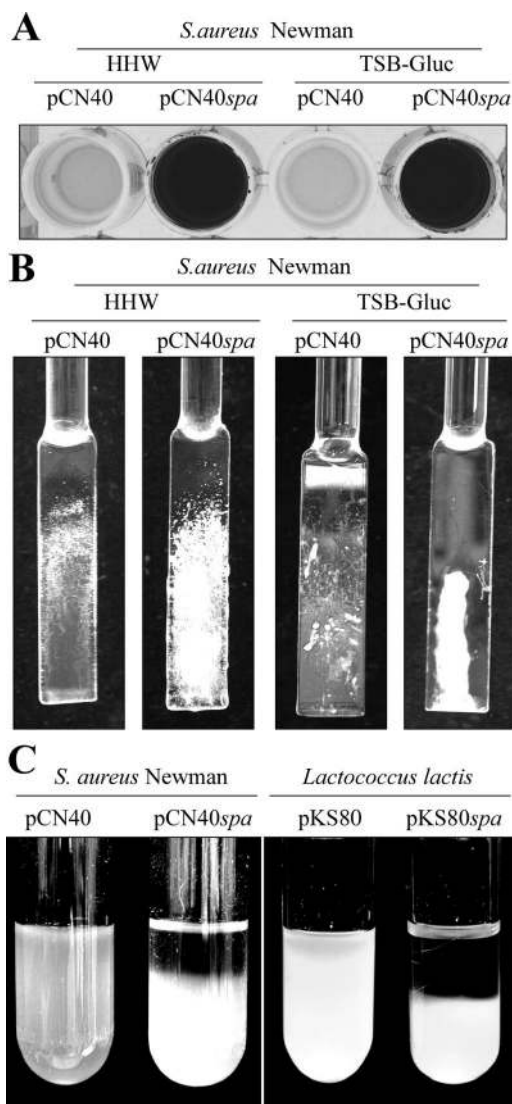

FIG. 5. (A) Biofilm formation phenotype in polystyrene microtiter dishes with *S. aureus* Newman wild-type strain complemented with the pCN40 control plasmid and pCN40*spa* plasmid carrying the *spa* gene under the P_{blaZ} promoter in HHWm and TSBg supplemented with 10 μg/ml of erythromycin. After 24 h of incubation, the microplates were washed and stained with crystal violet; the dye was dissolved in 200 μl of ethanol-acetone (80:20). (B) Biofilm development of *S. aureus* Newman wild-type strain with the pCN40 control plasmid and the pCN40*spa* plasmid expressing the *spa* gene under the P_{blaZ} promoter grown in a continuous flow of HHWm or TSBg for 24 h at 37°C. The glass slides where bacteria formed the biofilm are shown. (C) In vitro aggregation assay in glass tubes. On the left side is *S. aureus* Newman wild-type strain with the pCN40 control plasmid and the pCN40*spa* plasmid carrying the *spa* gene under P_{blaZ} promoter grown on HHWm supplemented with 10 μg/ml of erythromycin, and on the right side is *Lactococcus lactis* wild-type strain complemented with the pKS80 control plasmid and the pKS80*spa* plasmid grown on M17 supplemented with 10 μg/ml of erythromycin.

whether protein A needs to be anchored to the cell wall to promote multicellular behavior or if secreted protein A might be functional. We first examined the levels of protein A in the supernatants of ISP479r *agr*, ISP479r *agr* Δ*arlRS*, ISP479r *agr* Δ*arlRS* Δ*spa*, and ISP479r *agr* Δ*arlRS* Δ*spa* pCN40*spa*. Significant levels of protein A in the supernatants of the two biofilm-positive strains, ISP479r *agr* Δ*arlRS* and ISP479r *agr* Δ*arlRS* Δ*spa* pCN40*spa*, were confirmed (Fig. 7A). Supernatants of these bacterial strains, as well as pure commercial protein A and a supernatant containing a modified protein A lacking the LPXTG carboxy-terminal region, were added 1:1 (vol/vol) to ISP479r *agr* Δ*arlRS* Δ*spa* cultures. As shown in Fig. 7B, addition of pure protein A or supernatants containing protein A induced biofilm formation in the ISP479r *agr* Δ*arlRS* Δ*spa* strain, whereas protein A-deficient supernatants did not improve the biofilm formation capacity of this strain. Interestingly, the protein A derivative lacking the LPXTG domain retained the ability to induce biofilm formation. These results indicate that secreted protein A is fully functional in promoting biofilm development and suggest that protein A must interact with some compound of the bacterial surface to mediate biofilm development. To address this implication, we performed immunofluorescence microscopy analysis (Fig. 8). Protein A antiserum evenly bound to the cell surface of the ISP479r *agr*

Δ*arlRS* Δ*spa* strain when it was incubated with supernatants containing extracellular protein A, confirming that extracellular protein A interacts with the bacterial cell surface.

Expression of protein A contributes to subcutaneous catheter infection. To extend the relevance of our findings, we
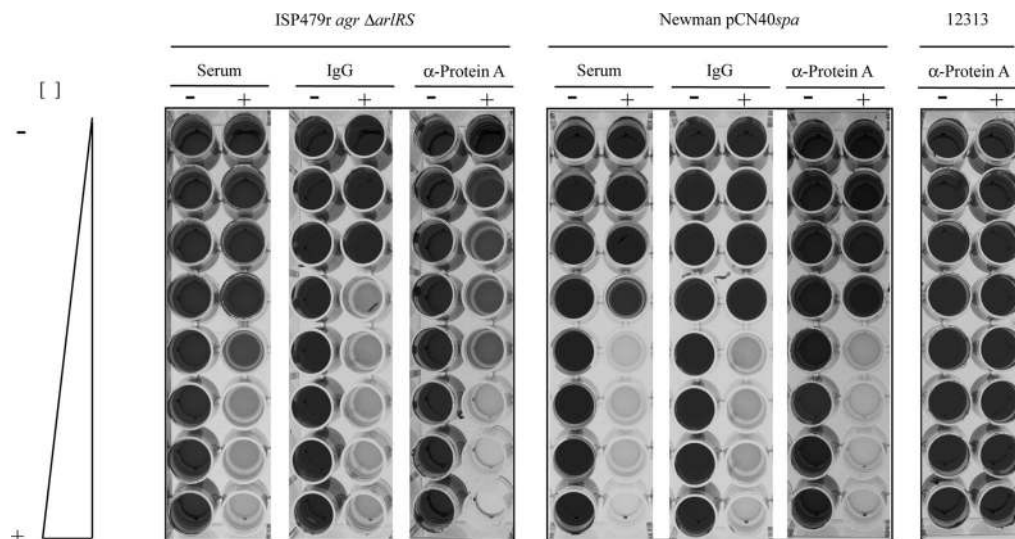
FIG. 6. Concentration-dependent inhibition of biofilm formation on microtiter plates. Increasing concentrations of unspecific rabbit antiserum, a purified IgG (Sigma), and specific anti-protein A antibodies (Sigma) were added to microtiter plates containing *S. aureus* ISP479r *agr* Δ*arlRS* strains, *S. aureus* Newman strain complemented with the pCN40*spa* plasmid carrying the *spa* gene under P_{blaZ} promoter, and *S. aureus* strain 12313. The nonspecific rabbit antiserum, commercial purified IgG, and anti-protein A-specific serum were used at concentrations ranging from 500 μg/ml to 3.6 μg/ml. HHWm was supplemented with 10 μg/ml of erythromycin as needed. After 24 h of incubation, the microplates were washed and stained with crystal violet.
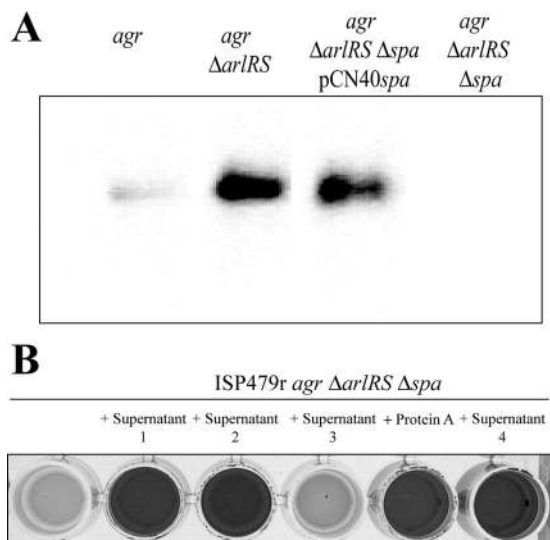

FIG. 7. (A) Western Blot analysis for protein A expression of the supernatants of ISP479r *agr*, ISP479r *agr* Δ*arlRS*, ISP479r *agr* Δ*arlRS* Δ*spa* complemented with the pCN40*spa*, and ISP479r *agr* Δ*arlRS* Δ*spa*. The cultures were grown overnight at 37°C in HHWm, and cells were collected by centrifugation at 12,000 rpm for 2 min (the medium was supplemented with 10 μg/ml of erythromycin as needed). The supernatants were resolved by SDS-PAGE in a 12% polyacrylamide gel and then subjected to Western blot analysis. The blot was probed with a 1:5,000 dilution of a peroxidase-conjugated AffiniPure rabbit anti-sheep IgG. (B) Biofilm formation phenotype of the ISP479r *agr* Δ*arlRS* Δ*spa* strain grown in the presence of supernatants obtained from ISP479r *agr* Δ*arlRS* (supernatant 1), ISP479r *agr* Δ*arlRS* Δ*spa* complemented with the pCN40*spa* (supernatant 2), ISP479r *agr* Δ*arlRS* Δ*spa* (supernatant 3), and ISP479r *agr* Δ*arlRS* Δ*spa* complemented with a truncated version of protein A that lacks the LPXTG domain (supernatant 4). Additionally, ISP479r *agr* Δ*arlRS* Δ*spa* was grown in the presence of pure commercial protein A (Sigma). The supernatants were added 1:1 to the culture medium, and the pure commercial protein A was added at a concentration of 10 μg/ml. After 24 h of incubation, the microplates were washed and stained with crystal violet. The dye was dissolved in 200 μl of ethanol-acetone (80:20).

investigated the effects of protein A on the capacity of *S. aureus* to colonize a subcutaneous foreign catheter. A 1-cm segment of an intravenous catheter was aseptically implanted in the subscapular space of mice, and animals were coinfected with equal numbers of *S. aureus* Newman and the corresponding protein A-deficient strain. The ratio of protein A$^+$ and protein A$^-$ clones recovered from infected catheters after 3 and 7 days was determined on agar plates with and without kanamycin. The results of two independent experiments are presented in Fig. 9. The number of protein A-positive colonies recovered from the catheters was approximately 10 times higher than protein A-negative colonies at day 3. Differences slightly increased at day 7 ($P < 0.005$, Student's *t* test). These data indicated that protein A significantly contributes to the development of biofilm-associated infections caused by *S. aureus*.

DISCUSSION

Protein A, the first surface protein of *S. aureus* to be identified, has served as a model for the characterization of the covalent anchorage of the LPXTG domain proteins by sortase A to the bacterial cell surface (40, 53, 54). Protein A is primarily known for its ability to bind to the Fc region of IgGs and inhibit opsonophagocytosis (14, 59). The consequence of this interaction is the coating of the bacterial surface with IgG molecules in the incorrect orientation to be recognized by the neutrophil Fc receptor (15). In addition, protein A can bind to the human von Willebrand factor, a large multimeric glycoprotein that mediates platelet adhesion at sites of endothelial damage to promote the appearance of blood clots (27, 46). Protein A also interacts with TNFR1 and stimulates an inflammatory response in airway epithelial cells (20, 21). In the present work, we have identified a novel function for protein A in the promotion of cell-to-cell interactions and biofilm formation by investigating the protein-dependent biofilm production
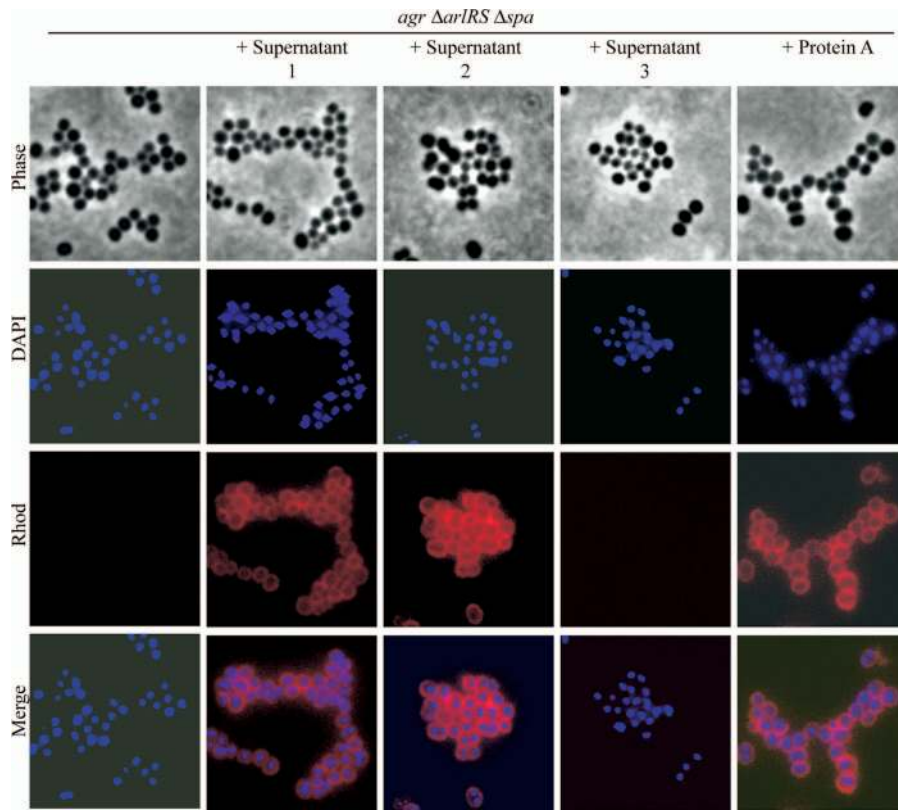
FIG. 8. Analysis of cell surface interactions of extracellular protein A by immunofluorescence microscopy. *S. aureus* ISP479r *agr* Δ*arlRS* Δ*spa* cells were incubated with supernatants of ISP479r *agr* Δ*arlRS* (supernatant 1), ISP479r *agr* Δ*arlRS* Δ*spa* pCN40*spa* (supernatant 2), and ISP479r *agr* Δ*arlRS* Δ*spa* (supernatant 3), as well as pure protein A. The samples were washed twice with PBS and fixed with 3% paraformaldehyde in PBS before incubation with a 1:200 dilution of a secondary anti-mouse IgG (whole molecule)–TRITC rabbit antibody (Sigma). The slides were mounted with Vectashield HardSet mounting medium with DAPI (Vector Laboratories) (see Materials and Methods for details). The experiment was repeated twice.

of an *agr arlRS* double mutant (56). The regulation of protein A by the *agr* system, and specifically by its effector molecule, RNA III, has been well-characterized. Accumulation of RNA III during the postexponential phase of growth represses *spa*

expression, not only at a transcriptional level but also by inhibiting its translation and inducing degradation of the stable *spa* mRNA by the double-strand-specific endoribonuclease III (RNase III) (6, 29, 43, 48). Thus, expression of protein A is
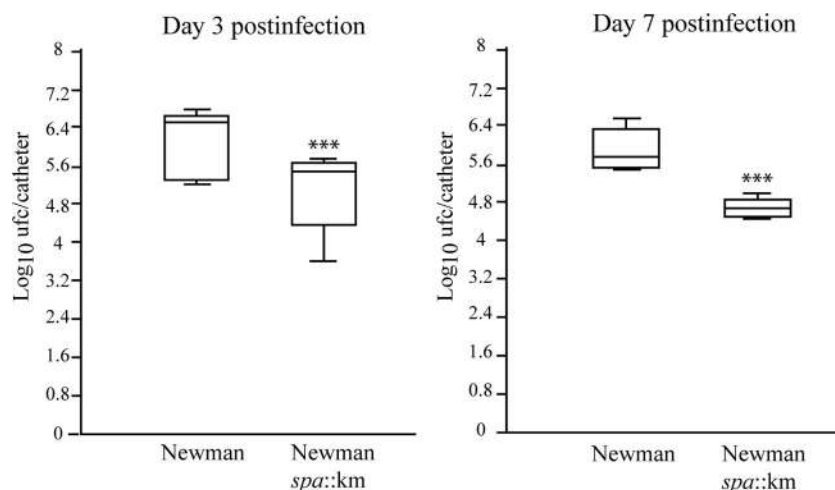

FIG. 9. A 1-cm segment of an intravenous catheter was aseptically implanted into the subcutaneous interscapular spaces of adult male mice. Each group of five mice was coinoculated with 10^5 CFU of both *S. aureus* Newman and the *spa* mutant strain (*spa*::*Km*). The figure shows the recovery from the implanted subcutaneous catheters with *S. aureus* Newman and the Newman *spa*::*Km* mutant. The boxes represent the means and standard deviations of two independent experiments. Significant differences between the wild-type and mutant strains are represented by asterisks ($P < 0.001$) at day 3 and day 7 postinfection. Bacteria were not detectable in control animals at the end of the experimental period.

induced at the exponential phase and repressed at the postexponential phase, when the expression of RNA III is maximal. In addition, *arlRS* has also been described as a repressor of *spa* expression, due to the fact that mutation in either of the two *arlRS* genes results in increased *spa* expression. The influence of *arlRS* on protein A expression requires the participation of *agr* or *sarA* regulators, as increased transcription was not observed in either an *agr* or *sarA* mutant strain (16). In agreement with these results, we did not observe significant upregulation of *spa* mRNA levels in *agr arlRS* mutants. However, the *agr arlRS* double mutant accumulates more protein A on the cell surface than the *agr* mutant, suggesting that *arlRS* either posttranscriptionally regulates *spa* mRNA stability or that decreased production of proteases due to the absence of *arlRS* indirectly provokes an increased accumulation of protein A. Whatever the molecular mechanism is, the final outcome of the double *agr arlRS* mutation is an increased accumulation of protein A on the bacterial surface. This accumulation together with the fact that most *S. aureus* strains are unable to produce PIA/PNAG exopolysaccharide in the HHWm synthetic medium has facilitated for us the observation that protein A can mediate biofilm formation. However, the effect of protein A on biofilm formation is not restricted to this double mutant. Thus, deletion of *spa* in wild-type strains reduces biofilm formation capacity in microfermentors, whereas it does not produce noticeable effects in microtiter plates, most likely because the amount of protein A under these conditions does not reach the threshold level required to promote cell-to-cell interactions and biofilm formation. In support of this hypothesis, we showed that overexpression of protein A or the increased accumulation of protein A in *agr* mutants induced biofilm development in microtiter plates. This last result correlates with previous data indicating that *agr*-deficient *S. aureus* mutants form more robust biofilms compared to wild-type strains (2, 3, 62, 64). Initially, it was proposed that the surfactant properties of δ-toxin encoded by RNA III limited the ability of the bacteria to form biofilms (62). More recently, it was shown that activation of the *agr* system through the addition of the AIP peptide at a physiological concentration represses biofilm development and triggers the detachment of established PIA/PNAG-independent biofilm matrix (3). The same study showed that the biofilm matrix produced by inactivation of the *agr* system was degraded by proteinase K and resistant to the treatment with dispersin B. Accordingly, *agr*-mediated biofilm detachment required the participation of extracellular proteases, as it did not occur in the presence of protease inhibitors or when the main protease encoding gene, aureolysin, was mutated (3). All these results strongly suggest that a proteinaceous compound is important for biofilm integrity and that *agr* activity promotes the detachment of a proteinaceous biofilm matrix. Our results support the idea that protein A is, at least in some strains, the proteinaceous compound responsible for biofilm development in *S. aureus agr* mutant strains.

Protein A-mediated aggregation and biofilm formation may result from homophilic interactions between two protein A molecules of neighboring cells. Such homophilic protein interactions leading to an autoaggregation phenotype have been described for protein H-mediated *Streptococcus pyogenes* cell aggregation (18), hemagglutinin-mediated cell aggregation in *Bordetella pertussis* (39), and antigen 43, which is involved in *E.*

coli biofilm formation (10). Alternatively, protein A could mediate heterophilic interactions with other surface proteins or even with a nonproteinaceous cell wall structure. In support of the former hypothesis, expression of protein A in *L. lactis* also induced aggregation, likely via heterophilic interactions with a compound that must be present on the cell wall of both bacteria. However, expression of protein A in *L. lactis* only induced aggregation of bacterial cells and was not sufficient to induce the development of a mature biofilm on microtiter plates or on the glass slide in microfermenters, suggesting that either additional factors are required for biofilm development or that *L. lactis* is unable to produce biofilm under the experimental conditions tested.

The covalent attachment of protein A to the bacterial surface is not required for its ability to promote cell-to-cell interactions. Thus, secreted protein A from *S. aureus* strains or a protein A variant lacking the carboxy-terminal LPXTG domain is sufficient to induce biofilm development. In contrast to Aap- or SasG-mediated biofilm development that must undergo proteolytic processing to render the proteins active for cell-to-cell interactions (7, 51), protein A induces biofilm development in an *agr arlRS* mutant that produces low levels of proteases. Indeed, it appears that the decreased production of extracellular proteases is at least partially responsible for the increased accumulation of protein A in this mutant and thereby proteolytic processing seems to not be required to activate protein A for biofilm development.

Several studies have demonstrated the role of protein A in staphylococcal virulence. Protein A-defective mutants show reduced virulence in murine models of septic arthritis, septicemia, and skin abscesses, most likely due to the antiphagocytic effect of protein A bound to the Fc fractions of the IgGs (47, 49). Protein A has also been shown to trigger apoptosis of murine B cells that express antigen receptors with VH3-Fab analogues, which likely contributes to immunosuppression during *S. aureus* infection (22). In the present study, coinfection experiments using a subcutaneous foreign device murine infection model showed that protein A significantly contributed to the development of biofilm-related infections. As these experiments were performed with a wild-type Newman strain and its corresponding protein A-deficient mutant, these results imply the existence of physiological situations in which the levels of protein A are sufficient to contribute to biofilm development. We cannot exclude the possibility that the increased susceptibility of protein A-defective mutants to phagocytosis might be negatively affecting the survival of the bacteria in the implanted catheter. However, if this were the case, we would expect that the number of recovered protein A-deficient bacteria would decrease throughout the duration of the experiment. However, similar numbers of protein A-deficient bacteria were recuperated at days 3 and 7, suggesting that survival of the bacteria does not decrease during the infection period.

In summary, our results indicate that two of the consequences of inactivation of the *agr* system, namely, overexpression of protein A and induction of biofilm formation capacity, are related. Whether the common observed *agr*-negative variants during the course of the infection (17, 52, 58) may lead to the establishment of bacterial interactions and biofilm formation to evade the immune system is an interesting hypothesis that warrants further experiments.

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