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Distinct Roles of Phenol-Soluble Modulins in Spreading of *Staphylococcus aureus* on Wet Surfaces

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The human pathogen *Staphylococcus aureus* is renowned for the rapid colonization of contaminated wounds, medical implants, and food products. Nevertheless, little is known about the mechanisms that allow *S. aureus* to colonize the respective wet surfaces. The present studies were therefore aimed at identifying factors used by *S. aureus* cells to spread over wet surfaces, starting either from planktonic or biofilm-associated states. Through proteomics analyses we pinpoint phenol-soluble modulins (PSMs) as prime facilitators of the spreading process. To dissect the roles of the eight PSMs produced by *S. aureus*, these peptides were chemically synthesized and tested in spreading assays with different *psm* mutant strains. The results show that PSMα3 and PSMγ are the strongest facilitators of spreading both for planktonic cells and cells in catheter-associated biofilms. Compared to the six other PSMs of *S. aureus*, PSMα3 and PSMγ combine strong surfactant activities with a relatively low overall hydropathicity. Importantly, we show that PSM-mediated motility of *S. aureus* facilitates the rapid colonization of wet surfaces next to catheters and the colonization of fresh meat.

Staphylococcus aureus is an opportunistic human pathogen that can cause a wide range of acute and chronic diseases, which range from superficial skin infections to life-threatening endocarditis and sepsis (1, 2). The ability of this Gram-positive bacterium to cause these infections depends on the production of secreted and cell wall-associated virulence factors. Of increasing concern is the ability of *S. aureus* to acquire resistance against antibiotics, as underscored by the global spread of methicillin-resistant *S. aureus* (MRSA) lineages.

Intriguingly, recent proteomics studies have revealed an enormous diversity in the production of virulence factors by different isolates of *S. aureus*, and only a few of these seem to be invariantly produced (3–5). Among the most commonly identified staphylococcal virulence factors, especially in the community-associated (CA)-MRSA lineages, are the so-called phenol-soluble modulins (PSMs) (6). These PSMs are short, amphipathic, α-helical peptides that have leukocidal activity and biosurfactant properties (7–9). The growth media of *S. aureus* cultures contain both N-terminally formylated and deformylated PSMs, suggesting that these virulence factors are substrates for the bacterial *N*-formylmethionine deformylase (9, 10).

To date, eight PSMs have been identified in *S. aureus*. These include the four PSMα1 to PSMα4 peptides (22 residues each), the PSMβ1 and PSMβ2 peptides (44 residues each), PSMγ (25 residues) and the recently reported PSM-mec (22 residues). The PSMα peptides are encoded by the *psm*α operon, the PSMβ peptides by the *psm*β operon, and PSMγ by the *hld* gene. Notably, the *hld* gene is embedded within the regulatory RNAIII molecule that is encoded by the *agr* locus. The gene for PSM-mec was identified in MRSA strains carrying the staphylococcal cassette chromosome *mec* (SCC*mec*) types II or III. The expression of all *psm* genes is controlled by the Agr system for quorum sensing (8, 11–13). This system modulates gene expression such that cell wall-associated virulence factors (e.g., the immunoglobulin G-binding protein A) are most highly expressed at low cell densities and that secreted virulence factors (e.g., the PSMs) are most highly expressed at high cell densities (14–21).

Notably, PSMs have been implicated in the high virulence of CA-MRSA lineages, which are readily transmitted by direct contact with a carrier (9, 22). The investigated CA-MRSA isolates produce higher amounts of the PSM peptides than the generally less virulent nosocomial MRSA isolates (9, 22). The PSMα peptides have the strongest leukolytic, proinflammatory, and chemotactic activities (9). Consistently, a strain lacking *psm*α had a decreased ability to cause skin lesions in mice and rabbits (9, 23). In addition to this, Wang et al. (24) have shown that the mortality rates and the levels of the inflammatory cytokine tumor necrosis factor alpha in the blood of mice infected with *psm*α or *psm*γ mutant strains were substantially reduced. The PSMβ peptides appear less important for cytolysis and inflammation but, in low concentrations, they seem to promote biofilm formation by *Staphylococcus epidermidis*. High amounts of the same PSMβ peptides do, however, promote the detachment of staphylococcal cells from biofilms both *in vitro* and *in vivo* (24).

Although *S. aureus* was originally believed to be nonmotile, recent studies have shown that this organism is capable of spreading over wet surfaces (25–27). We have previously shown that a mix of the four PSMα peptides can promote this so-called colony spreading phenotype (27), and we hypothesized that this relates to their strong surfactant properties (9). The Agr system is an impor-

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
DH5α	λ$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	29
TOP10	Cloning host for TOPO vector; F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$ ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen Life Technologies
S. aureus		
RN4220	Restriction-deficient derivative of NCTC8325, cured of all known prophages	30
NCTC8325	HA-MSSA strain, *agr*$^+$ Δ*rsbU*	31
NCTC8325$^-$	HA-MSSA strain, Δ*agr* Δ*rsbU*	27
NCTC8325 Δ*psm*α	NCTC8325 lacking the *psm*α genes	This study
NCTC8325 Δ*psm*β	NCTC8325 lacking the *psm*β genes	This study
NCTC8325 Δ*psm*α Δ*psm*β	NCTC8325 lacking the *psm*α and *psm*β genes	This study
SH1000	NCTC8325-4 derivative, *rsbU*$^+$ *agr*$^+$	32
SH1000$^-$	NCTC8325-4 derivative, *rsbU*$^+$ Δ*agr*	27
SH1000 Δ*psm*α	SH1000 lacking the *psm*α genes	This study
SH1000 Δ*psm*β	SH1000 lacking the *psm*β genes	This study
SH1000 Δ*psm*α Δ*psm*β	SH1000 lacking the *psm*α and *psm*β genes	This study
HG001	NCTC8325 derivative, *rsbU*$^+$ *agr*$^+$	33
HG001$^-$	NCTC8325 derivative, *rsbU*$^+$ Δ*agr*	27
HG001 Δ*psm*α	HG001 lacking the *psm*α genes	This study
HG001 Δ*psm*β	HG001 lacking the *psm*β genes	This study
HG001 Δ*psm*α Δ*psm*β	HG001 lacking the *psm*α and *psm*β genes	This study
Newman	ATCC 25904; high-level clumping factor production; σ^{B+}	34
Newman Δ*agr*	Newman derivative, Δ*agr*::*tetM*	35
Newman Δ*psm*α	Newman lacking the *psm*α genes	This study
Newman Δ*psm*β	Newman lacking the *psm*β genes	This study
Newman Δ*psm*α Δ*psm*β	Newman lacking the *psm*α and *psm*β genes	This study
LAC USA300	CA-MRSA	9
LAC USA300 Δ*psm*α	LAC USA300 lacking the *psm*α genes	9
Plasmids		
pUC18	Ap^r; ColE1, ϕ80d*lacZ*; *lac* promoter	36
TOPO	pCR-Blunt II-TOPO vector; Km^r	Invitrogen Life Technologies
pMAD	*E. coli*/*S. aureus* shuttle vector that is temperature sensitive in *S. aureus* and contains the *bgaB* gene; Em^r Ap^r	37
pRIT5H	*E. coli*/*S. aureus* shuttle vector; Cm^r; *spa* promoter	38
pSW4-GFPopt	pSW4 plasmid containing 729-bp AseI/BamHI-cloned *gfpopt*	39
GFPopt-pRIT5H	pRIT5H with *gfpopt* gene; Ap^r Km^r	This study
pAH9	*sarA* promoter P_i-RFP	40

[a] CA, community acquired; HA, hospital acquired; MRSA, methicillin-resistant *S. aureus*. Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance.

tant determinant for colony spreading due to its control over the synthesis of PSMs (27). However, Agr is also needed for biofilm formation, which gives it a decisive role in the choice between motile and sessile lifestyles of *S. aureus*. Furthermore, it was shown that covalently cell wall-anchored proteins, like FnbpA, FnbpB, ClfA, and ClfB, can set a limit to the colony-spreading ability of *S. aureus* cells (28). To date, very little was known about the roles of individual PSM peptides in colony spreading and whether these are the main factors promoting spreading. Therefore, the present studies were aimed at dissecting the roles of the different PSM peptides in colony spreading. Furthermore, we wanted to test whether N-terminally formylated and deformylated PSMs are equally potent in colony spreading. To achieve these objectives, we constructed multiple *psm* mutant strains, which were then incubated in the presence or absence of synthetic PSMs. Importantly, our results show that PSMα3 and PSMγ have key roles in colony spreading. Furthermore, our observations link PSMα3 and PSMγ to the spreading of staphylococcal cells from catheter-related biofilms, and they suggest that PSM-mediated spreading plays a major role in the movement of *S. aureus* over biotic surfaces. Importantly, our findings show that the PSMs that most effectively promote the spreading of *S. aureus* combine high surfactant activities with low overall hydropathicity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids that were used in this study are listed in Table 1. *Escherichia coli* strains were grown in Lysogeny broth (LB) at 37°C under vigorous shaking. *S. aureus* strains were grown in tryptic soy broth (TSB) at 37°C under vigorous shaking, or on tryptic soy agar (TSA) plates. Where necessary,

antibiotics were added at the following concentrations: ampicillin, 100 μg/ml (for *E. coli*); erythromycin, 5 μg/ml (for *S. aureus*); and chloramphenicol, 10 μg/ml (for *S. aureus*).

Colony spreading assay. The colony-spreading assay was performed essentially as described by Kaito et al. (25), but with minor previously described modifications (27). To detect colony spreading of the *S. aureus* strains SH1000 and Newman along the growth curve, these strains were grown in TSB for 24 h. Samples were collected at hourly intervals for the first 7 h and after 24 h of growth. All samples were immediately tested for colony spreading. Equal amounts of cells from each time point were spotted onto the 0.24% TSA plates. All spreading assays were repeated at least five times.

Mass spectrometric analyses of culture supernatants. Strains Newman (*agr*$^+$, Δ*agr*, or Δ*psm*α), LAC USA300 (*agr*$^+$ or Δ*psm*α), NCTC8325 (*agr*$^+$ or Δ*agr*) and HG001 (*agr*$^+$ or Δ*agr*) were grown in TSB. At an optical density at 600 nm (OD_{600}) of 2 and 6, 3-ml culture samples were collected, and cells were separated from the growth medium by centrifugation (8,000 × *g*, 4°C, 10 min). To precipitate the secreted proteins, the medium fractions were incubated at 4°C with 10% trichloroacetic acid overnight. The proteins were pelleted (20,000 × *g*, 4°C, 20 min), washed with acetone, and subsequently dissolved in 8 M urea. Protein concentrations were determined using the Bio-Rad DC protein assay according to the protocol of the supplier. The samples (2 μg) were reduced with 10 mM dithiothreitol (DTT; Duchefa Biochemie) for 30 min and alkylated with 10 mM iodoacetamide (Sigma-Aldrich) for another 30 min in the dark. Finally, the protein samples were incubated overnight at 37°C with 40 ng of trypsin (Promega). Peptides were purified using ZipTips (4), separated and analyzed by liquid chromatography-tandem mass spectrometry using an Easy-nLCII high-pressure liquid chromatography system (Thermo Fisher Scientific, Waltham, MA) coupled directly to an LTQ Orbi-Trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The Easy-nLCII was equipped with a self-packed analytical column (C18-material [Luna 3u C18(2)100A; Phenomenex], 100-μm inner diameter by 200-mm column). Peptide elution was performed by application of a binary gradient of buffer A (0.1% [vol/vol] acetic acid) and B (99.9% [vol/vol] acetonitrile, 0.1% [vol/vol] acetic acid) over a period of 80 min with a flow rate of 300 nl/min. The mass spectrometry (MS) analyses were performed as described by Miller et al. (41). A nonredundant database was constructed that contained all available *S. aureus* protein sequences (UniProt) that differ in at least one amino acid residue (see Table S2 and FASTA Files S1 and S2 in the supplemental material). This database was subsequently used for the database search (including a concatenated reversed database, 40,098 entries). The database search was performed with Sequest using a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10 ppm. Oxidation of methionine and carbamidomethylation of cysteine were specified in Sequest as variable modifications. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold (version Scaffold 3.3.2; Proteome Software, Inc., Portland, OR). Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of >0.10 and XCorr scores of greater than 1.9, 2.2, 3.8, and 3.8 for singly, doubly, triply, and quadruply charged peptides. All experiments have been conducted in independent duplicates. Proteins were only accepted as being identified if they were detected in both biological replicates per sample set. With these filter parameters, the false-positive rate was below 1%.

Construction of a GFP expressing vector. For constitutive expression of GFP in *S. aureus* the *gfpopt* gene was amplified from plasmid pSW4-GFPopt using primers GFPoptFR and GFPoptRV (see Table S3 in the supplemental material). The amplified PCR product was then cloned in plasmid pRIT5H using the EcoRI and SalI restriction sites. Expression of GFP was detected using the IVIS Spectrum from Caliper Life Sciences using the specific filter for GFP (excitation, 465 nm; emission, 520 nm).

Construction of PSM mutant strains of *S. aureus*. Mutants of *S. aureus* were constructed using the temperature-sensitive plasmid pMAD (37) and previously described procedures (42). Primers were designed using the genome sequence of *S. aureus* NCTC8325 (http://www.ncbi.nlm.nih.gov/nuccore/NC007795). To delete the *psm*α and/or *psm*β operons, the primer pairs with the designations F1/R1 and F2/R2 were used for PCR amplification of the respective upstream and downstream regions (each ~500 bp) (see Table S3 in the supplemental material). The R1 and F2 primers contain a 24-bp linker sequence to fuse the flanking regions by PCR prior to cloning in pMAD. The resulting plasmids were used to transform *S. aureus* strain RN4220 via electroporation. Next, the plasmids were isolated from the RN4220 strain and used to transform the *S. aureus* SH1000, HG001, NCTC8325, or Newman strains via electroporation in order to delete their *psm*α and/or *psm*β operons through subsequent plasmid integration and excision steps (43). At the end of the procedure, white colonies were screened for the absence of the *psm*α and/or *psm*β genes by colony PCR using primers F1 and R2.

Complementation of *psm* mutations by synthetic PSM peptides and determination of surfactant properties. The PSMα1 to PSMα4, PSMβ1 and PSMβ2, PSMγ, and PSM-mec peptides were synthesized as described previously with a C-terminal four-residue glycyl spacer and an ε-amino biotinyl lysine (27). All peptides were dissolved in dimethyl sulfoxide plus 10 mM DTT to a concentration of 12 mM. The peptides were then diluted 10-fold in phosphate-buffered saline, and 2 μl of each peptide solution was spotted in the center of a soft TSA plate prior to the inoculation of the *S. aureus* mutant strain to be tested for spreading. The surfactant properties of the PSMs were determined by spotting 2 μl of each peptide solution in the center of a soft TSA plate and by subsequently measuring the diameter of the resulting transparent halo.

Colony spreading of *S. aureus* from catheter-associated biofilms. Catheters were positioned on soft agar plates and 2 μl of bacteria grown overnight were spotted next onto these catheters before the plates were incubated overnight at 37°C. Subsequently, the catheters were transferred to fresh soft TSA plates and incubated again overnight at 37°C, which resulted in the formation of catheter-associated biofilms. Catheters with associated biofilms were transferred to fresh soft TSA plates, where 2 μl of PSM peptide solution was spotted prior to the transfer of the catheters. The plates were then incubated overnight at 37°C. Images were recorded with a G:box (Syngene, Leusden, Netherlands).

Colony spreading of *S. aureus* on pork. Pieces of pork meat were placed in sterile petri dishes and, approximately in the center, 2 μl of bacteria grown overnight in TSB were spotted. The meat was incubated at 37°C for 48 h, after which images were recorded with a Sony cyber-shot camera.

RESULTS

Secreted factors regulated by *agr* are responsible for colony spreading. We have previously shown that the *agr* locus, which regulates the synthesis of secreted virulence factors, is required for colony spreading of *S. aureus*. This suggested that secreted virulence factors are of prime importance for colony spreading. Another indication for the role of secreted factors in spreading came from the observation that colonies of *S. aureus* cells that are capable of spreading were surrounded by a transparent halo (Fig. 1A). This halo was absent from nonspreading *agr* mutant colonies. Furthermore, when overnight grown *agr*-proficient bacteria were spotted on soft agar plates, spreading of the cultured cells was visible after a few seconds, suggesting that any secreted factors needed for spreading were already present in the culture. In fact, this area of rapid spreading remained clearly visible on the plates (marked by a box in Fig. 1B), which was due to the emergence of subsequent "waves" of spreading cells from the initial spreading zone. This suggested that the factors needed for spreading were not synthesized continuously. To investigate whether the spreading factors were synthesized growth phase dependently, *S. aureus* cells were grown to different growth stages and tested for the rapid

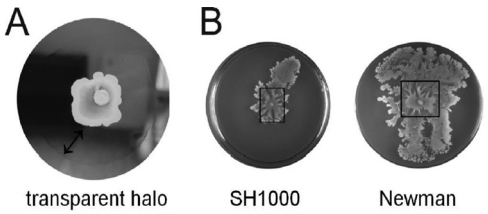

FIG 1 Characteristic features of *S. aureus* spreading motility. (A) A transparent "halo" is produced by cells of spreading colonies. A similar halo is also generated by surfactants that are spotted on soft agar plates (not shown). (B) Spreading motility involves a rapid phase of spreading by the cells spotted on a plate. Subsequently, waves of cells emerge from the cells in the initial spreading zone. The boxes mark the rapid spreading zones that emerged from the sites of inoculation of strains SH1000 and Newman on soft agar plates.

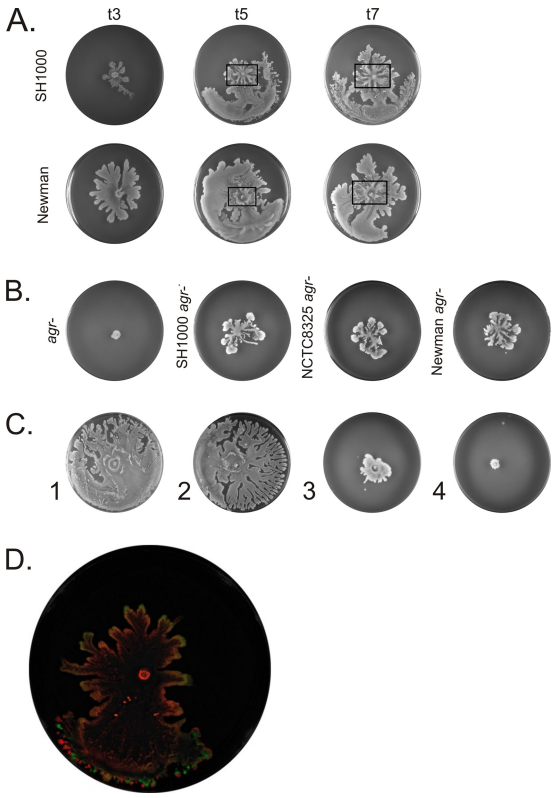

FIG 2 Colony spreading depends on the growth phase of inoculated *S. aureus* cells and on secreted factors produced by agr^+ strains. (A) Colony spreading by planktonic cells of *S. aureus* strains SH1000 or Newman collected from cultures in different growth stages; t3 corresponds to the early exponential growth phase (OD_{600} = 1.5), t5 to the late exponential phase (OD_{600} = 6.8), and t7 to early stationary phase (OD_{600} = 9.0). (B) Filter-sterilized culture medium of agr^+ cells of *S. aureus* strain Newman promotes rapid spreading of Δ*agr* cells of strains SH1000, NCTC8325, and Newman. As shown for Δ*agr* cells of strain SH1000, no spreading is observed when fresh medium is used (left plate labeled "*agr*−"). (C) Culture supernatants of agr^+ cells need to be applied on top of soft agar plates to promote spreading. Fresh soft agar plates were prepared and inoculated as follows: plate 1, regular soft agar with the wild-type strain Newman; plate 2, regular soft agar with the wild-type strain Newman resuspended in 2 μl of filter-sterilized supernatant of the wild-type strain Newman; plate 3, 200 μl of filter-sterilized supernatant of the wild-type strain Newman included within the soft agar prior to inoculation with the wild-type strain Newman; and plate 4, 200 μl of filter-sterilized supernatant of the wild-type strain Newman included in the soft agar prior to inoculation with Δ*agr* cells of strain Newman. (D) Δ*agr* cells carrying plasmid GFPopt-pRIT5H for expression of GFP were coinoculated with agr^+ cells carrying plasmid pAH9 for expression of mCherry. GFP fluorescence of the Δ*agr* cells is detectable at the edges of the spreading zone (green color), whereas mCherry fluorescence of the agr^+ cells (red color) is detectable in the entire area covered by spreading. All spreading assays were repeated at least five times.

spreading phenotype. Indeed, the rapid spreading was not observed for cells in the early exponential growth phase (Fig. 2A, time point t3), but it started when the cells reached the late exponential phase (t5) and continued in the stationary phase (t7). Consistent with our previous findings, these time points correspond with the activation of the Agr system (17). To verify that secreted factors promote colony spreading, Δ*agr* cells from different strains were resuspended in filtered supernatants of overnight grown agr^+ strains and the cell suspension was spotted on top of soft agar plates. As shown in Fig. 2B, the filtered supernatants were able to promote colony spreading of the Δ*agr* cells. Notably, when the filtered supernatant of an agr^+ strain was included within the soft agar, it did not promote the colony spreading of Δ*agr* cells (Fig. 2C, plate 4). Together, these results demonstrate that secreted factors regulated via the Agr system are both needed and sufficient for colony spreading of *S. aureus*. Importantly, to promote colony spreading, these secreted factors need to be present on the surface of the soft agar on which the bacteria spread.

As the spent growth medium of agr^+ cells can promote the spreading of Δ*agr* cells, we wondered whether the Δ*agr* cells would also spread if they were grown in the presence of agr^+ cells. To test this, an overnight culture of a nonspreading Δ*agr* derivative of *S. aureus* HG001 expressing the green fluorescent protein (GFP) was mixed with an overnight culture of the authentic *S. aureus* HG001 strain (agr^+) expressing the fluorescent mCherry protein. Next aliquots of the mixed culture were transferred to soft agar plates and incubated overnight. Images were taken using the IVIS spectrum with the specific filters for GFP (excitation, 465 nm; emission, 520 nm) and mCherry (excitation, 570 nm; emission, 620 nm) to monitor the spreading of the two different strains. GFP was only detected at the edges of the colony spreading area, indicating that the Δ*agr* strain was very well able to spread over the soft agar together with the agr^+ strain (Fig. 2D). Since the Δ*agr* HG001 strain cannot spread by itself, this observation implies that nonspreading *S. aureus* cells can passively spread on the soft agar plates with the help of factors that are actively secreted by spreading *S. aureus* cells.

MS identification of secreted proteins potentially involved in colony spreading. To investigate which proteinaceous factors are involved in colony spreading, culture supernatants of agr^+, Δ*agr*, and Δ*psm*α variants of the *S. aureus* strains Newman, LAC USA300, NCTC8325, and HG001 were analyzed using MS. As expected, the MS analyses revealed many strain-dependent differences between the investigated *S. aureus* strains (see Table S1 in the supplemental material). Importantly, the only proteins that were common in the media of all tested agr^+ strains and absent from the media of all tested Δ*agr* strains were PSMα2, PSMα3, PSMα4, PSMβ1, PSMβ2, and the staphylococcal lipase 1. Only the identified PSM peptides have potential surfactant properties that might promote colony spreading, and therefore we focused all our following studies on these peptides.

Dissection of PSM function with synthetic peptides reveals major roles for PSMα3 and PSMγ in colony spreading. Surfactants lower the surface tension of liquid-to-air interfaces, and due to this activity they facilitate the motility of many different bacte-

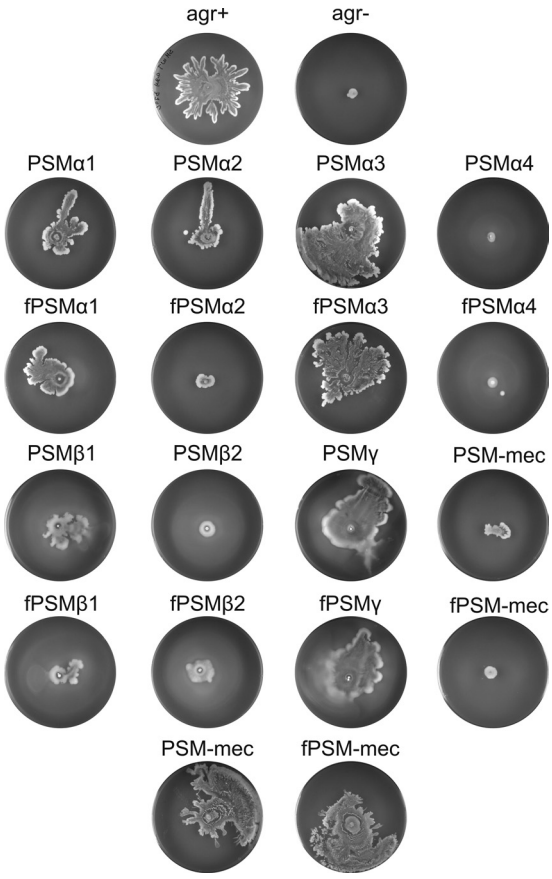

FIG 3 Particular synthetic PSM peptides facilitate spreading of Δ*agr* cells. To identify factors that facilitate spreading of Δ*agr* cells (labeled "agr−" in the figure), chemically synthesized PSMs from *S. aureus* (plate rows 2 to 5) were spotted in the center of soft agar plates prior to the inoculation with *S. aureus* SH1000 Δ*agr* cells. Both N-terminally formylated PSMs (marked with an "f" prefix) and nonformylated peptides were used in the assay. In addition, the PSM-mec peptides were tested for a potentially inhibitory role in spreading by cells of the *S. aureus* SH1000 *agr*$^+$ strain (bottom row). All spreading assays were repeated at least five times.

rial species (44–46). To determine which of the previously identified PSM peptides might be involved in the spreading of *S. aureus*, we tested synthetic PSMα1-4, PSMβ1-2, PSMγ, and PSM-mec peptides for their ability to promote colony spreading. To this end, all PSMs were individually spotted at the same concentrations on soft agar plates. This analyses showed that the PSMα3 and PSMγ peptides strongly promoted the colony spreading by otherwise nonspreading Δ*agr* strains (Fig. 3). Other PSMs—such as PSMα1, PSMα2, and PSMβ1—promoted colony spreading to lesser extents, whereas PSMα4, PSMβ2, and PSM-mec did not promote spreading at all (Fig. 3). Based on genetic studies, it was previously proposed that PSM-mec might inhibit colony spreading (47). We therefore tested the synthetic PSM-mec peptide for possible inhibitory effects, but unfortunately, no such effects were detectable in our experimental setting (Fig. 3, plates in bottom row). Furthermore, our studies show that N-terminally formylated PSMs promote spreading equally well as the nonformylated peptides (Fig. 3). It thus seems that both the formylated and nonformylated forms of PSMα3 and PSMγ contribute to the movement of *S. aureus* cells over wet surfaces. Importantly, both forms of PSMα3 and PSMγ exerted their effect on spreading in a dose-dependent manner (see Fig. S1 in the supplemental material). This underscores the view that these two PSMs actively contribute to colony spreading.

To investigate why different PSMs promote the spreading of *S. aureus* cells to different extents, we compared their surfactant properties on soft agar plates by spotting 2 μl of each peptide solution in the center of such a plate (without adding bacteria) and by subsequently measuring the diameter of the resulting transparent halo. This revealed that PSMα3, PSMγ, and PSMβ2 have the strongest surfactant properties (Table 2). Furthermore, we assessed other potentially relevant parameters of the investigated PSMs, namely, the grand average of hydropathicity (GRAVY), the hydrophobic moment (μH), the net charge (z), the number of polar residues plus glycine, and the number of nonpolar residues. The results show that the PSMs that most effectively promote the spreading of *S. aureus*, namely, PSMα3 and PSMγ, combine high surfactant activities with low hydropathicity (Table 2). The other investigated PSM properties seem to be of minor relevance for spreading activity. Notably, while PSMβ2 has relatively strong surfactant properties, it has a high hydropathicity, suggesting that this high hydropathicity may counteract the promotion of spreading. Consistent with this view, PSM-mec combines the lowest surfactant properties with the highest overall hydropathicity (Table 2).

Genetic dissection of PSM function reveals additive effects in colony spreading. As underscored by our proteomics analyses, *agr*-deficient strains are completely defective in the synthesis of PSMs. To identify the contributions of the *psmα* and *psmβ* operons to spreading, the respective single- and double-mutant strains were constructed. Next, the spreading ability of these mutants was compared to the parental strain and to the equivalent *agr* mutant, which is fully PSM deficient. To quantify spreading activity, all images of spreading assays were analyzed with ImageJ, and the area covered by the cells was determined. Figure 4 shows that

TABLE 2 Properties of *S. aureus* PSM peptides[a]

Parameter[a]	PSMα1	PSMα2	PSMα3	PSMα4	PSMβ1	PSMβ2	PSMγ	PSM-mec
Surfactant properties (mean length [cm] ± SD)	1.0 ± 0.4	1.9 ± 0.1	7.4 ± 0.5	0.6 ± 0.1	1.4 ± 0.4	4.3 ± 2.4	6.3 ± 0.4	0.6 ± 0.1
Grand avg of hydropathicity (GRAVY)	0.957	0.890	0.305	1.700	0.570	0.607	0.150	1.100
Hydrophobic moment, <μH>	0.551	0.562	0.563	0.599	0.308	0.214	0.587	0.530
Net charge z	2	3	2	2	−1	−1	−1	−1
Polar residues + GLY (no./%)	10/47.62	10/47.62	10/45.45	6/30.00	23/52.27	22/50.00	14/53.85	10/45.45
Nonpolar residues (no./%)	11/52.38	11/52.38	12/54.55	14/70.00	21/47.73	22/50.00	12/46.15	12/54.55

[a] Surfactant properties were determined by spotting 2 μl of a PSM solution (6 to 1.2 mM) on a soft agar plate and measuring the diameter of the resulting transparent halo. The grand average of hydropathicity (GRAVY) was determined using the ProtParam tool provided by the ExPASy server. The hydrophobic moment (μH), the net charge (z), the amount of polar residues + GLY, and the amount of nonpolar residues were obtained by using Heliquest (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py).

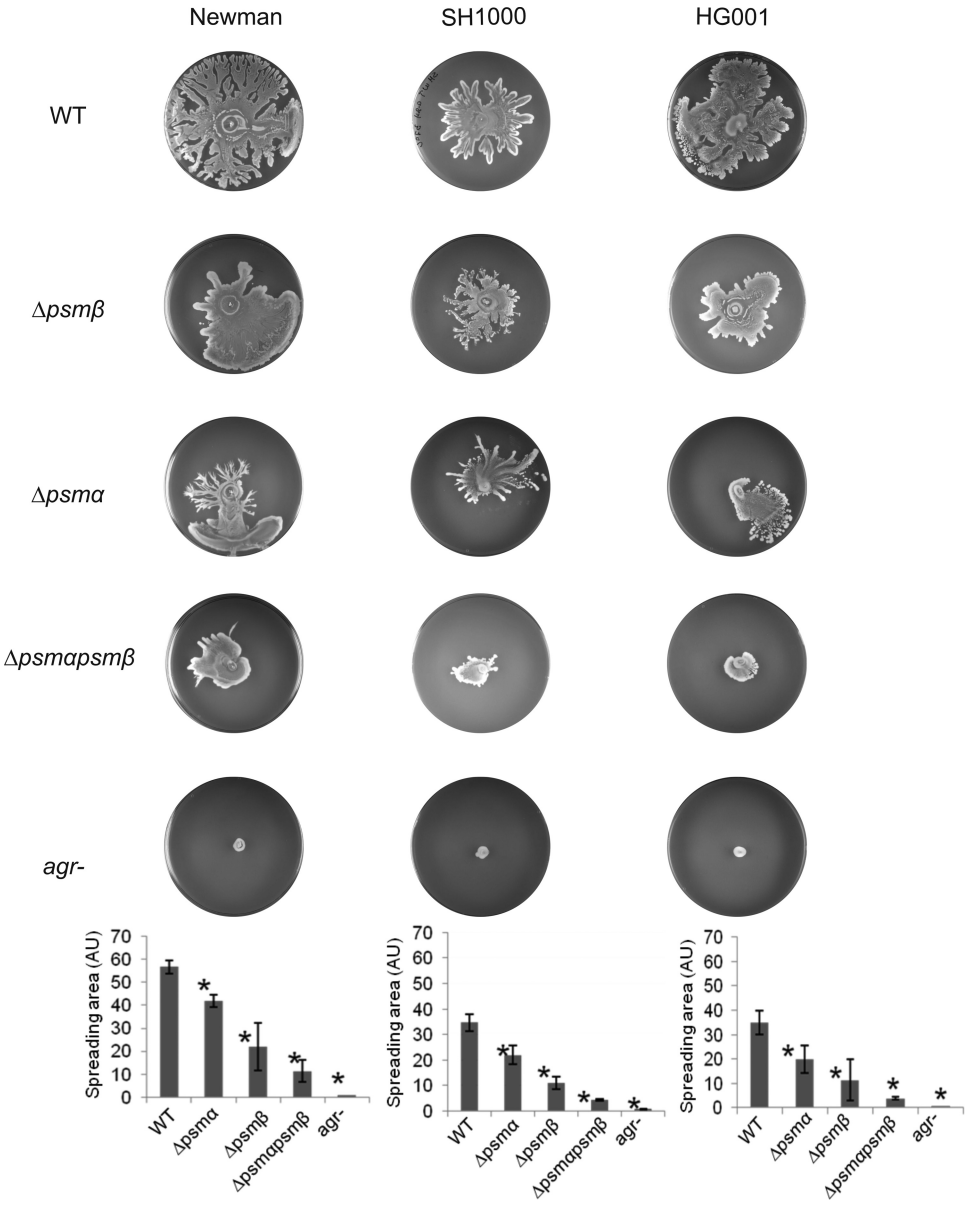

FIG 4 Additive effects of *psm*α and *psm*β gene deletions on the spreading of *S. aureus* cells. The *psm*α and/or *psm*β loci of the *S. aureus* strains Newman, SH1000, or HG001 were deleted, and the effects on colony spreading were compared with the effects of an *agr* mutation. Subsequently, the spreading areas of the investigated mutant and parental strains were determined by ImageJ, and statistical analyses were performed based on triplicate measurements for each individual strain. The graphs show the areas covered in arbitrary units (AU).

deletion of the *psm*β operon had a moderate but reproducible effect on spreading. Deletion of the *psm*α operon had a more severe effect on spreading and the spreading activity of the *psm*α *psm*β double mutant was even lower (Fig. 4). Notably, the spreading activity of the double mutant was still higher than that of the *agr* mutant, which suggests that the remaining activity of the double mutant is due to the production of PSMγ. This view is consistent with the above finding that the synthetic PSMγ is sufficient to restore spreading of *agr* mutant strains (Fig. 3). A comparison of the results obtained for the single and double *psm*α *psm*β mutant variants of strains Newman, SH1000, and HG001 shows that the deletion of the *psm*α operon has the strongest negative impact on spreading, which is fully consistent with the findings obtained with the synthetic peptides added at equimolar concentrations. Furthermore, our genetic dissection of the function of PSM-encoding loci shows that they contribute additively to colony spreading.

Spreading activity of *S. aureus* cells from catheter-associated biofilms or on pork meat. PSMs allow *S. aureus* to move across wet surfaces. In the assays described above this was demonstrated starting with planktonic cells growing in a broth. However, we wanted to know whether spreading would be detectable also in assays that mimic clinically relevant surfaces and conditions. In one approach, we therefore tested whether *S. aureus* cells present in a catheter-associated biofilm have the ability to spread. As shown in Fig. 5, both *agr*$^+$ and Δ*agr* strains were able to form

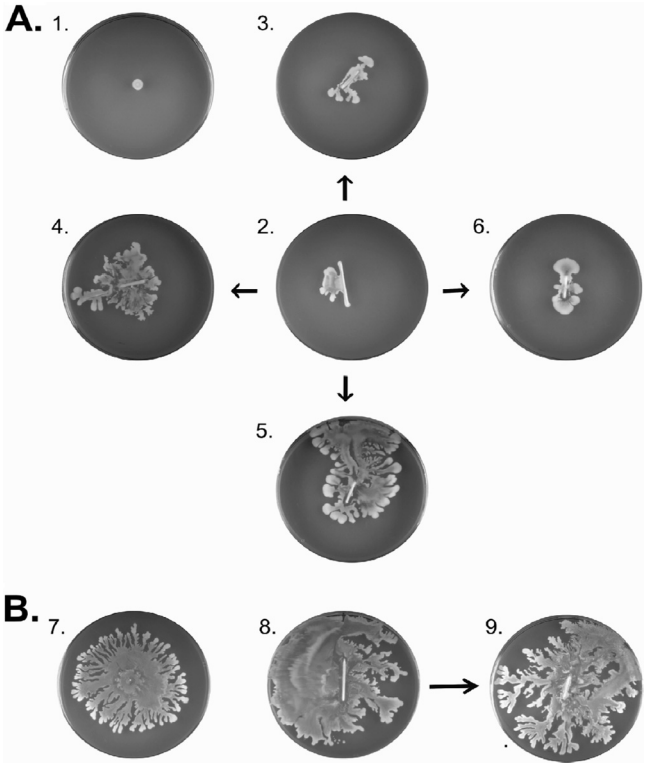

FIG 5 The spread of *S. aureus* cells from catheter-associated biofilms is facilitated by particular PSM peptides. (A) To investigate whether the spreading of cells from a catheter-associated biofilm is facilitated by PSMs, biofilms of Δ*agr* cells of *S. aureus* Newman were grown on ~1-cm-long strips of catheter material. These strips were then incubated on soft agar plates under differing conditions. Plate 1, 1egative control plate, showing that the used *S. aureus* Newman Δ*agr* strain is unable to spread; plate 2, biofilms of Δ*agr* cells were grown on catheter strips placed on a soft agar plate as shown with plate 2; plate 3, catheter strip with a biofilm (as on plate 2) transferred to a fresh soft TSA plate without further additions. Some "outgrowth" of the cells is observed but no spreading. Plate 4, catheter strip with a biofilm (as on plate 2) transferred to a soft TSA plate to which the PSMα3 peptide was added prior to the positioning of the catheter strip; plate 5, catheter strip with a biofilm transferred to a plate with the PSMγ peptide (as in plate 4); plate 6, catheter strip with a biofilm transferred to a plate with the PSMβ1 peptide (as in plate 4). (B) Control experiments with agr^+ cells of *S. aureus* Newman. Plate 7, positive control plate showing colony spreading of agr^+ cells; plate 8, colony spreading of Newman agr^+ cells from a catheter strip; plate 9, catheter strip from plate 8 transferred to a fresh soft TSA plate without added PSMs. All spreading assays were repeated at least five times.

biofilms on catheter material (plates 8 and 2, respectively). Interestingly, when the catheters were transferred to fresh TSA plates, the agr^+ strains were well able to detach and spread away from the catheter material (plate 9). This phenomenon was not observed for the tested Δ*agr* strains (plate 3). Importantly, however, when these catheters were transferred to fresh plates on which 2 μl of PSMα3 or PSMγ were spotted beforehand, the Δ*agr* strains were also able to detach and spread away from the catheter (plates 4 and 5, respectively). In contrast, the PSMβ1 did not facilitate the spreading of Δ*agr* cells from the catheter material (plate 6). These findings suggest that the ability of PSMs to promote spreading is important for *S. aureus* to move away from a biofilm and to colonize the surrounding wet surface. This idea was further tested by studying colony spreading on fresh pork meat. As predicted, agr^+ cells were substantially more efficient than Δ*agr* cells in colonizing pieces of pork meat upon 48 h of incubation at 37°C. In the experiment shown in Fig. 6A, the agr^+ cells colonized an area that was on average ~2.5-fold larger than the area colonized by Δ*agr* cells. Importantly, the spreading phenotypes of *psm* mutant strains on fresh pork meat (Fig. 6B) resembled by-and-large the respective spreading phenotypes on soft agar plates (Fig. 4). While *psm*β mutant cells were not significantly inhibited in their spreading on pork, the *psm*α mutant cells showed a significant spreading defect. Spreading on pork meat was most severely affected by deletion of both the *psm*α and *psm*β genes, even to a slightly higher extent than *agr* mutant cells. It thus seems that PSM-mediated colony spreading has a general role in the colonization of wet surfaces by *S. aureus*.

DISCUSSION

Colony spreading and PSMs. The present studies have focused attention on the role of secreted factors that are needed for the rapid colony spreading phenotype of *S. aureus*. The first indication for an important role of secreted factors in colony spreading was the presence of a transparent halo around the expanding colonies. Subsequent analyses showed that culture supernatants of spreaders were sufficient to make nonspreaders move on a wet soft agar surface and that the main common components in the media of spreaders were PSMs. Importantly, culture supernatants of spreaders facilitated the spreading of nonspreaders only when these supernatants were added on top of the soft agar. This is in accordance with the apparent need for high surfactant properties of the PSMs in order to be able to promote spreading. In contrast, when the culture supernatants of spreaders were included within the soft agar, they were unable to promote the spreading of nonspreaders and they even seemed to inhibit the spreading by spreaders (Fig. 2C). With synthetic PSM peptides we subsequently demonstrated conclusively that several PSMs of *S. aureus* are sufficient to promote colony spreading of otherwise nonspreading strains. This view was confirmed by mutagenesis experiments in which the *psm*α and/or *psm*β operons were deleted. Taken together, our present findings show that PSMα3 and PSMγ are the key players in colony spreading, and that the other PSMα's and PSMβ's have minor roles in spreading. We observed no role for the PSM-mec neither in the promotion nor the inhibition of spreading. The latter observations are intriguing, because it has been reported that the PSM-mec and/or the PSM-mec mRNA can inhibit colony spreading (47). Since the synthetic PSM-mec peptides gave no phenotype, whereas other synthetic PSM peptides were active, it seems most likely that the previously reported effects do not relate to a translated product but rather to a regulatory effect of the *psm-mec* gene. We were unable to assess this possibility with the strains used in our studies, because they lack type II or type III SCC*mec* elements that encode PSM-mec. In any case, it is safe to conclude from our studies that addition of the PSM-mec peptide did not interfere with the function of the main spreading-promoting PSMα3 and PSMγ peptides. Indeed, an independent study confirms that deletion of the *psm-mec* gene does not affect the production of the other PSMs compared to the respective parental strains (11). Lastly, it was previously reported that *S. aureus* secretes both N-terminally formylated and deformylated PSM peptides. Our present data show that the removal of the N-terminal formyl group has no consequences with respect to the activity of PSMs in colony spreading. This indicates that the sur-

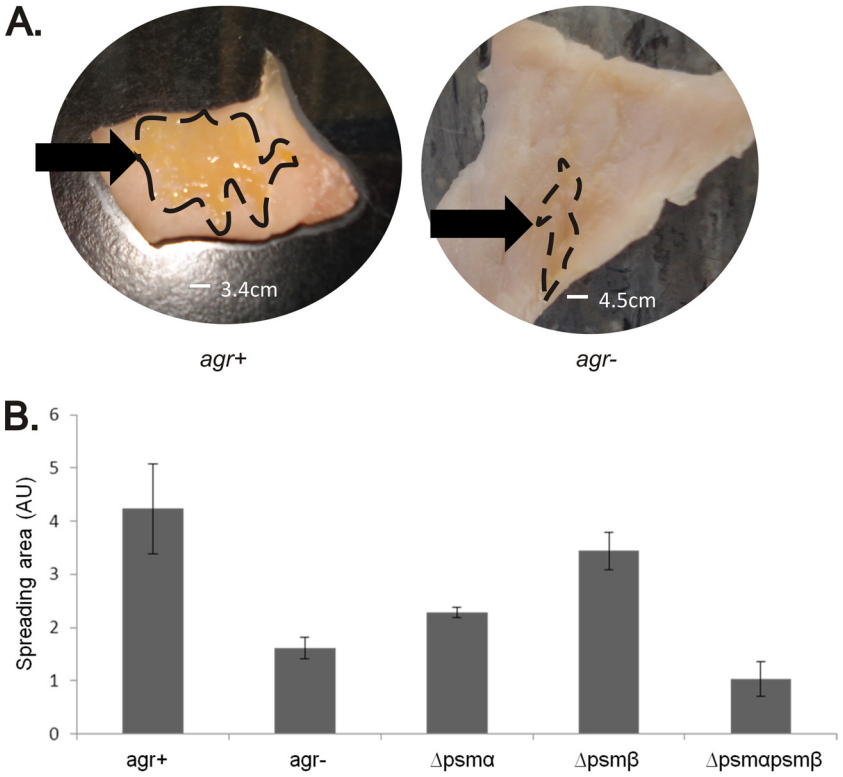

FIG 6 Spreading of *S. aureus* on meat. (A) Overnight grown *S. aureus* SH1000 *agr*$^+$ or Δ*agr* (labeled "*agr*−" in the figure) cells were spotted on pork meat, which was subsequently incubated 48 h at 37°C. SH1000 *agr*$^+$ cells covered a 2.5-fold larger area than SH1000 Δ*agr* cells. The spreading areas are marked with dashed lines. (B) Spreading areas of *agr*$^+$ and Δ*agr S. aureus* SH1000 cells, and single or double *psm*α and/or *psm*β mutant strains upon spotting onto fresh pork meat. All spreading assays were repeated at least three times.

factant properties of PSM peptides are not substantially influenced by N-terminal formylation or deformylation.

At present, we do not know at what concentrations exactly the different PSMs need to be present locally to promote spreading. In this respect, one has to bear in mind that both the synthetic and the natural PSM peptides investigated in the present studies will diffuse not only over the soft agar surface but also into the agar underneath the surface. In addition, in a regular spreading assay, the *S. aureus* cells applied to a soft agar plate will initially make use of the PSMs present in the growth medium, which explains the first observed wave of "rapid" spreading. Subsequently, these cells will produce additional PSMs, allowing them to spread further over the plate. Therefore, it is very difficult to make statements about local concentrations of the PSM peptides on plates and how these correlate with the concentrations present in the spent medium. The situation is somewhat different when synthetic PSM peptides are used for a spreading assay, since this essentially represents the first wave of 'rapid' spreading. To this end, relatively high amounts of PSM peptides were initially tested. However, as shown by dose response curves with the PSMs that promote effective spreading (see Fig. S1 in the supplemental material), we can mimic effective spreading with concentrations that are only ~10-fold higher than the concentrations measured in the growth medium (9). We believe that this is a realistic situation, because bacteria that are spreading with the aid of PSMs secreted into the growth medium can employ a mix of several PSMs for spreading. Moreover, the Agr-deficient cells that were used for the spreading assays cannot produce any PSMs by themselves in the course of an assay, while Agr-proficient cells will produce their own PSMs. In this context it is important to note that when the concentrations of PSMs were stepwise increased, higher spreading levels were achieved. This mimics in some way the situation where Agr-proficient cells produce several "waves of PSMs" during the course of a colony spreading experiment.

Is colony spreading by *S. aureus* clinically relevant? A key question in the analysis of colony spreading is whether this property is clinically relevant. As a first approach to answer this question, we recently tested the spreading ability of 500 different clinical isolates that are representative for invasive *S. aureus* infections in Europe. More than 85% of these strains were able to spread (our unpublished observations). While this does not tell us that spreading was important for the actual infections, this finding does show that spreading is a very common feature of strains that have caused invasive infections in humans. Accordingly, it is conceivable that invasive strains make use of their spreading ability to move away from catheters and other implanted devices so that they can efficiently colonize wet surfaces of the human body. Clearly, our experiments with catheter material show that *S. aureus* cells originating form a biofilm have a similar spreading ability as planktonic cells. In this light it is not surprising that we find the same PSMs (i.e., PSMα3 and PSMγ) to be most effective in both types of assays. These findings therefore suggest that spreading may be a clinically relevant staphylococcal trait.

It has been observed that most clinical *S. aureus* isolates are *agr*$^+$, but Δ*agr* isolates are also isolated from patients. This population heterogeneity is likely to be advantageous for *S. aureus* since

agr$^+$ strains are more potent in initiating infections (48), while the Δ*agr* strains are more potent in the establishment of chronic infections through biofilm formation (49). Our finding that nonspreading cells can "hitch-hike" along with the spreaders on soft agar plates would suggest that a similar phenomenon might "help" Δ*agr* cells also in the colonization of wet surfaces, either in the human host or in other habitats. One of these other habitats might be animal meat intended for human consumption. There are many reports on the contamination of meat products with *S. aureus*, including MRSA (50–52). This is highly unwanted, first, because *S. aureus* is renowned as a causative agent of food poisoning (53). In addition, the contamination of food products with *S. aureus*, MRSA in particular, is a high-risk factor for frail and immunocompromised individuals who are more susceptible for staphylococcal infections. As shown by our experiments with pork, *agr*$^+$ cells can colonize larger surfaces of the investigated meat in shorter periods of time than Δ*agr* cells, reflecting the differences in their spreading ability on soft agar plates. Also, spreading on pork meat was reduced when the *psm*α genes were deleted, and it was even more reduced when both the *psm*α and *psm*β genes were deleted. Thus, we hypothesize that spreading is an important parameter at least in food spoilage and, consequently, in food poisoning by *S. aureus*. It will remain a challenge for future studies to verify this hypothesis and to pinpoint any other potentially clinically relevant roles of staphylococcal spreading.

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REFERENCES

1. **Lowy FD.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339**: 520–532.
2. **Peacock SJ, de Silva I, Lowy FD.** 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. **9**:605–610.
3. **Ziebandt AK, Kusch H, Degner M, Jaglitz S, Sibbald MJ, Arends JP, Chlebowicz MA, Albrecht D, Pantucek R, Doskar J, Ziebuhr W, Broker BM, Hecker M, van Dijl JM, Engelmann S.** 2010. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. Proteomics **10**:1634–1644.
4. **Dreisbach A, Hempel K, Buist G, Hecker M, Becher D, van Dijl JM.** 2010. Profiling the surfaceome of *Staphylococcus aureus*. Proteomics **10**: 3082–3096.
5. **Dreisbach A, van Dijl JM, Buist G.** 2011. The cell surface proteome of *Staphylococcus aureus*. Proteomics **11**:3154–3168.
6. **Otto M.** 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu. Rev. Microbiol. **64**:143–162.
7. **Queck SY, Jameson-Lee M, Villaruz AE, Bach TH, Khan BA, Sturdevant DE, Ricklefs SM, Li M, Otto M.** 2008. RNAIII-independent target gene control by the *agr* quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. Mol. Cell **32**:150–158.
8. **Queck SY, Khan BA, Wang R, Bach TH, Kretschmer D, Chen L, Kreiswirth BN, Peschel A, DeLeo FR, Otto M.** 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS Pathog. **5**:e1000533. doi:10.1371/journal.ppat.1000533.
9. **Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. **13**:1510–1514.
10. **Somerville GA, Cockayne A, Durr M, Peschel A, Otto M, Musser JM.** 2003. Synthesis and deformylation of *Staphylococcus aureus* delta-toxin are linked to tricarboxylic acid cycle activity. J. Bacteriol. **185**:6686–6694.
11. **Chatterjee SS, Chen L, Joo HS, Cheung GY, Kreiswirth BN, Otto M.** 2011. Distribution and regulation of the mobile genetic element-encoded phenol-soluble modulin PSM-*mec* in methicillin-resistant *Staphylococcus aureus*. PLoS One **6**:e28781. doi:10.1371/journal.pone.0028781.
12. **Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Novick RP.** 1994. Glycerol monolaurate inhibits the production of beta-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. J. Bacteriol. **176**:4204–4209.
13. **Sibbald MJ, Ziebandt AK, Engelmann S, Hecker M, de Jong A, Harmsen HJ, Raangs GC, Stokroos I, Arends JP, Dubois JY, van Dijl JM.** 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol. Mol. Biol. Rev. **70**:755–788.
14. **George EA, Muir TW.** 2007. Molecular mechanisms of *agr* quorum sensing in virulent staphylococci. Chembiochem **8**:847–855.
15. **Morfeldt E, Janzon L, Arvidson S, Lofdahl S.** 1988. Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. Mol. Gen. Genet. **211**:435–440.
16. **Novick RP, Geisinger E.** 2008. Quorum sensing in staphylococci. Annu. Rev. Genet. **42**:541–564.
17. **Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12**:3967–3975.
18. **Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesch F, Moghazeh S.** 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. **248**:446–458.
19. **Peng HL, Novick RP, Kreiswirth B, Kornblum J, Schlievert P.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **170**:4365–4372.
20. **Recsei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, Novick RP.** 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar. Mol. Gen. Genet. **202**:58–61.
21. **Ziebandt AK, Becher D, Ohlsen K, Hacker J, Hecker M, Engelmann S.** 2004. The influence of *agr* and sigmaB in growth phase-dependent regulation of virulence factors in *Staphylococcus aureus*. Proteomics **4**:3034–3047.
22. **Kobayashi SD, DeLeo FR.** 2009. An update on community-associated MRSA virulence. Curr. Opin. Pharmacol. **9**:545–551.
23. **Li M, Cheung GY, Hu J, Wang D, Joo HS, DeLeo FR, Otto M.** 2010. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. J. Infect. Dis. **202**:1866–1876.
24. **Wang R, Khan BA, Cheung GY, Bach TH, Jameson-Lee M, Kong KF, Queck SY, Otto M.** 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J. Clin. Invest. **121**:238–248.
25. **Kaito C, Sekimizu K.** 2007. Colony spreading in *Staphylococcus aureus*. J. Bacteriol. **189**:2553–2557.
26. **Kaito C, Omae Y, Matsumoto Y, Nagata M, Yamaguchi H, Aoto T, Ito T, Hiramatsu K, Sekimizu K.** 2008. A novel gene, *fudoh*, in the SCCmec region suppresses the colony spreading ability and virulence of *Staphylococcus aureus*. PLoS One **3**:e3921. doi:10.1371/journal.pone.0003921.
27. **Tsompanidou E, Sibbald MJ, Chlebowicz MA, Dreisbach A, Back JW, van Dijl JM, Buist G, Denham EL.** 2011. Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J. Bacteriol. **193**:1267–1272.
28. **Tsompanidou E, Denham EL, Sibbald MJ, Yang XM, Seinen J, Friedrich AW, Buist G, van Dijl JM.** 2012. The sortase A substrates FnbpA, FnbpB, ClfA, and ClfB antagonize colony spreading of *Staphylococcus aureus*. PLoS One **7**:e44646. doi:10.1371/journal.pone.0044646.
29. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
30. **Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, Novick RP.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305**:709–712.
31. **Novick R.** 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology **33**:155–166.
32. **Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ.** 2002.

sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. J. Bacteriol. **184**:5457–5467.
33. **Pohl K, Francois P, Stenz L, Schlink F, Geiger T, Herbert S, Goerke C, Schrenzel J, Wolz C.** 2009. CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. J. Bacteriol. **191**: 2953–2963.
34. **Duthie ES, Lorenz LL.** 1952. Staphylococcal coagulase; mode of action and antigenicity. J. Gen. Microbiol. **6**:95–107.
35. **Wolz C, McDevitt D, Foster TJ, Cheung AL.** 1996. Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64**: 3142–3147.
36. **Norrander J, Kempe T, Messing J.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
37. **Arnaud M, Chastanet A, Debarbouille M.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. Appl. Environ. Microbiol. **70**:6887–6891.
38. **Kuroda M, Ito R, Tanaka Y, Yao M, Matoba K, Saito S, Tanaka I, Ohta T.** 2008. *Staphylococcus aureus* surface protein SasG contributes to intercellular autoaggregation of *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **377**:1102–1106.
39. **Sastalla I, Chim K, Cheung GY, Pomerantsev AP, Leppla SH.** 2009. Codon-optimized fluorescent proteins designed for expression in low-GC Gram-positive bacteria. Appl. Environ. Microbiol. **75**:2099–2110.
40. **Boles BR, Horswill AR.** 2008. *agr*-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathog. **4**:e1000052. doi:10.1371/journal.ppat.1000052.
41. **Miller M, Dreisbach A, Otto A, Becher D, Bernhardt J, Hecker M, Peppelenbosch MP, van Dijl JM.** 2011. Mapping of interactions between human macrophages and *Staphylococcus aureus* reveals an involvement of MAP kinase signaling in the host defense. J. Proteome Res. **10**:4018–4032.
42. **Kouwen TR, Trip EN, Denham EL, Sibbald MJ, Dubois JY, van Dijl JM.** 2009. The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. Antimicrob. Agents Chemother. **53**:4702–4711.
43. **Sibbald MJ, Winter T, van der Kooi-Pol MM, Buist G, Tsompanidou E, Bosma T, Schafer T, Ohlsen K, Hecker M, Antelmann H, Engelmann S, van Dijl JM.** 2010. Synthetic effects of *secG* and *secY2* mutations on exoproteome biogenesis in *Staphylococcus aureus*. J. Bacteriol. **192**:3788–3800.
44. **Kearns DB, Losick R.** 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. **49**:581–590.
45. **Kohler T, Curty LK, Barja F, van Delden C, Pechere JC.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182**:5990–5996.
46. **Lindum PW, Anthoni U, Christophersen C, Eberl L, Molin S, Givskov M.** 1998. *N*-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. J. Bacteriol. **180**:6384–6388.
47. **Kaito C, Saito Y, Nagano G, Ikuo M, Omae Y, Hanada Y, Han X, Kuwahara-Arai K, Hishinuma T, Baba T, Ito T, Hiramatsu K, Sekimizu K.** 2011. Transcription and translation products of the cytolysin gene *psm-mec* on the mobile genetic element SCCmec regulate *Staphylococcus aureus* virulence. PLoS Pathog. **7**:e1001267. doi:10.1371/journal.ppat.1001267.
48. **Traber KE, Lee E, Benson S, Corrigan R, Cantera M, Shopsin B, Novick RP.** 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology **154**:2265–2274.
49. **Yarwood JM, Schlievert PM.** 2003. Quorum sensing in *Staphylococcus* infections. J. Clin. Invest. **112**:1620–1625.
50. **van Loo IH, Diederen BM, Savelkoul PH, Woudenberg JH, Roosendaal R, van Belkum A, Lemmens-den Toom N, Verhulst C, van Keulen PH, Kluytmans JA.** 2007. Methicillin-resistant *Staphylococcus aureus* in meat products, the Netherlands. Emerg. Infect. Dis. **13**:1753–1755.
51. **O'Brien AM, Hanson BM, Farina SA, Wu JY, Simmering JE, Wardyn SE, Forshey BM, Kulick ME, Wallinga DB, Smith TC.** 2012. MRSA in conventional and alternative retail pork products. PLoS One **7**:e30092. doi:10.1371/journal.pone.0030092.
52. **Kluytmans JA.** 2010. Methicillin-resistant *Staphylococcus aureus* in food products: cause for concern or case for complacency? Clin. Microbiol. Infect. **16**:11–15.
53. **Le Loir Y, Baron F, Gautier M.** 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. **2**:63–76.