Published in final edited form as:

Nat Microbiol. 2019 July ; 4(7): 1114–1119. doi:10.1038/s41564-019-0413-x.

Resistance to leukocytes ties benefits of quorum-sensing dysfunctionality to biofilm infection

Lei He^{1,2}, Katherine Y. Le^{1,3}, Burhan A. Khan^{1,#}, Thuan H. Nguyen¹, Rachelle L. Hunt¹, Justin S. Bae¹, Juraj Kabat⁴, Yue Zheng¹, Gordon Y. C. Cheung¹, Min Li², and Michael Otto^{1,*}

¹Pathogen Molecular Genetics Section, Laboratory of Bacteriology, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, 50 South Drive, Bethesda, Maryland 20814, USA

²Department of Laboratory Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, No. 160 Pujian Road, Shanghai 200127, China

³Division of Hospital Internal Medicine, Department of Medicine, Mayo Clinic College of Medicine, 200 1st Street SW, Rochester, Minnesota 55902, USA

⁴Biological Imaging Section, Research Technologies Branch, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, 4 Memorial Drive, Bethesda, Maryland 20814, United States

Abstract

Social interactions play an increasingly recognized key role in bacterial physiology¹. One of the best studied is quorum sensing (QS), a mechanism by which bacteria sense and respond to the status of cell density². While QS is generally deemed crucial for bacterial survival, QS-dysfunctional mutants frequently arise in in-vitro culture. This has been explained by the fitness cost an individual mutant, a “quorum cheater”, saves at the expense of the community³. QS mutants are also often isolated from biofilm-associated infections, including cystic fibrosis lung infection⁴, as well as medical device infection and associated bacteremia^{5–7}. However, despite the frequently proposed use of QS blockers to control virulence⁸, the mechanisms underlying QS dysfunctionality during infection have remained poorly understood. Here we show that in the major human pathogen *Staphylococcus aureus*, QS-dysfunctional mutants arise exclusively in biofilm infection, while in non-biofilm-associated infection there is a high selective pressure to maintain QS control. We demonstrate that this infection-type dependence is due to QS-

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use:http://www.nature.com/authors/editorial_policies/license.html#terms

*Correspondence and lead contact: motto@niaid.nih.gov.

Author contributions

L.H., K.Y.L. and R.L.H. performed confocal microscopy. B.A.K. performed in-vitro quorum cheating assays. L.H., T.H.N., G.Y.C.C., J.S.B., R. L.H. and Y.Z. performed animal experiments. L.H., T.H.N. and R.L.H. performed leukocyte killing assays. L.H. performed all other experiments. L.H., K.Y.L., G.Y.C.C., J.K., J.S.B., and M.O. analyzed the data. M.L and M.O. designed and supervised experiments. M.O. wrote the paper.

#Present address: University of Queensland Diamantina Institute, Translational Research Institute, 37 Kent Street, Woolloongabba, Queensland 4102, Australia

Competing interest statement

The authors have no competing interests to disclose.

dysfunctional bacteria having a significant survival advantage in biofilm infection, because they form dense and enlarged biofilms that provide resistance to phagocyte attacks. Our results link the benefit of QS-dysfunctional mutants in vivo to biofilm-mediated immune evasion, thus to mechanisms that are specific to the in-vivo setting. Notably, our findings explain why QS mutants are frequently isolated from biofilm-associated infections and provide guidance for the therapeutic application of QS blockers.

The benefits of QS dysfunctionality (quorum cheating) have so far almost exclusively been investigated using in-vitro experimentation. To our knowledge, only two studies addressed the fate of QS-dysfunctional isolates (quorum cheaters) in vivo, by using mixtures of wild-type and isogenic QS-deficient strains for infection. One study performed a *Pseudomonas aeruginosa* wound infection⁹ and another an *S. aureus* wax moth (*Galleria mellonella*) larva virulence model¹⁰. Essentially, both studies concluded that the dynamics of in-vivo QS functionality follow the in-vitro social cheating model, inasmuch as results were explained by exploitation of cooperative signaling and production of common-good secreted factors by QS cheaters, rather than by host interaction^{9,10}. Thus, despite this previous use of in-vivo infection models, in-vivo QS dysfunctionality has not been investigated mechanistically in light of the host environment and the presence of host defenses. Furthermore, the rise of spontaneous QS mutants has not been directly assessed in an animal infection model. Finally, it has not been addressed why QS mutants preferentially arise in biofilm-associated types of infection.

To analyze the role and development of QS dysfunctionality during infection, considering both biofilm-associated and non-biofilm-associated infection, we chose the major human pathogen *S. aureus* as subject of study, because it is a leading cause of both infection types¹¹. *S. aureus* has one well-described QS system called accessory gene regulator (Agr), which controls the production of virtually all secreted degradative enzymes and immune evasion factors such as toxins¹². In addition, Agr promotes biofilm maturation¹³. Combining cytolytic and surfactant activities, the amphiphilic phenol-soluble modulins (PSM) peptides are key effectors of staphylococcal QS, facilitating both biofilm maturation as well as toxin-mediated pathogenesis^{14–16}. Several previous in-vitro observations suggested potential mechanisms to explain occurrence and in-vivo benefits of Agr-dysfunctional mutants in a clinical setting¹⁷. These included findings of an inverse correlation of Agr expression and antibiotic resistance¹⁸, increased fitness cost of Agr over-expression triggered by selected antibiotics¹⁹, in addition to our previous observations of extended biofilm formation by *agr* mutants^{5,14,20}. However, the function of Agr dysfunctionality has not been directly investigated in vivo. In that regard, it is of particular importance that according to previous research by Shopsin et al., *agr* mutants do not transfer between patients²¹. Thus, the rise of *S. aureus* QS dysfunctionality is not a population-wide process, but occurs anew in every individual infection, meaning that its investigation in an animal infection model well reflects the real-life scenario.

We first measured the extent of the development of QS-dysfunction in *S. aureus* in vitro as a basis for comparison with our in-vivo study. To that end, we monitored the rise of Agr-dysfunctional bacteria in nine different methicillin-resistant *S. aureus* (MRSA) strains and

one laboratory strain by serial passage of bacterial cultures. QS-dysfunctional isolates developed at considerable rates in almost all of the ten strains (Supplementary Fig. 1). In some strains, QS dysfunctionality was found to temporarily reach almost 100% of the bacterial population. Then, we frequently detected the “rebound effect” predicted from the selective pressure that arises due to the fact that in a population completely devoid of QS-controlled secreted degradative enzymes, bacteria cannot acquire essential nutrients³. In accordance with the notion that QS-controlled enzymes are needed to degrade essential food sources, growth of a culture of exclusively QS-deficient (*agr*) bacteria was significantly impaired in comparison to isogenic wild-type bacteria (Supplementary Fig. 1). Thus, in accordance with established models, in-vitro QS dysfunctionality is widespread and considerable in extent in *S. aureus*, but a complete “take over” by quorum cheaters is prevented due to the growth defects of entirely QS-deficient communities.

We then proceeded to the main purpose of our study, the investigation of QS functionality in vivo. To that end, we used two mouse infection models, a device-related, biofilm-associated infection model, and a non-biofilm-associated abscess model. We chose the abscess model as example of a non-biofilm-related model due to its otherwise high similarity to the device-related model in terms of infection site and kinetics^{16,22}. We used the methicillin-resistant (MRSA) strain LAC (pulsed-field type USA300), because USA300 isolates (i) highly express *Agr*²³, thus representing an optimal choice to investigate *Agr*-dependent differences, and (ii) were the most frequent causes of *S. aureus* infections, including device-associated infections, in the U.S., in the last decade^{24,25}. In the non-biofilm model, *Agr*-functional (wild-type) bacteria demonstrated significant survival advantages over isogenic *agr*-negative bacteria (Fig. 1a). Furthermore, pathogenesis in terms of abscess size was clearly enhanced in the presence of *Agr* (Supplementary Fig. 2). These findings are in accordance with the fact that *Agr* regulates a plethora of factors specifically needed for in-vivo pathogenesis^{26,27}, including the PSMs, which showed a significant impact on abscess size and bacterial survival (Fig. 1a, Supplementary Fig. 2). Importantly, we did not find any QS-dysfunctional mutants in > 1000 tested bacteria isolated from 7-day old abscesses caused by the wild-type strain (Tab. 1). These findings indicate that rise of *Agr* dysfunctionality is strongly suppressed during *S. aureus* non-biofilm infection. This is in stark contrast to the in-vitro data we obtained and indicates that the importance of QS-controlled factors that are specific to survival during infection in vivo supersedes the effects that allow the rise of quorum cheaters in vitro.

Prompted by the studies that have reported the isolation of QS-dysfunctional mutants from chronic and biofilm-associated staphylococcal infections on indwelling medical devices^{5,6} and associated bloodstream infections^{7,28}, we then analyzed the development and consequences of *Agr* dysfunctionality in a mouse model of biofilm-associated infection. We chose a model of subcutaneous device-associated infection, as this model is well established and pathogenesis is known to depend on biofilm formation on the subcutaneously placed catheter²⁹. In contrast to the non-biofilm-associated abscess model, the isogenic *agr* and *psm* mutants had significant survival advantages over the wild-type strain on the implanted device (Fig. 1b). These results are in accordance with our previous findings demonstrating *Agr*- and PSM-mediated impact on biofilm expansion in vitro¹⁴, but which so far had not been verified for in-vivo relevance. Importantly, we detected the development of QS mutants

at a frequency of $\sim 5 \times 10^{-3}$ over the 1-week duration of the infection, which represents a significant difference to the absence of QS mutants in the abscess model ($p=0.0196$, Fisher's exact test) (Tab. 1). All mutations were non- or missense point mutations that occurred in the *agrA* or *agrC* genes (Supplementary Tab. 1), which represent the previously identified main sites of *agr* mutations in clinical strains⁶.

As in this commonly used biofilm infection model, the infection heals within about one week, the expected further expansion of the initial rise of QS mutants cannot be directly measured. Therefore, we used mixed wild-type/*agr* mutant (1:1) inocula and measured the fate of QS mutants by determining the wild-type/mutant percentage in the biofilm and non-biofilm infection models after one week (Fig. 1c,d). Strikingly, the percentage of *agr* mutants in the non-biofilm skin infection model dropped from 50% to $0.3 \pm 0.2\%$, providing further evidence that during non-biofilm infection, functionality of Agr is imperative and quorum cheaters are strongly selected against (Fig. 1e). In stark contrast, in the device-associated biofilm infection model, the observed percentage of *agr* mutants after one week of infection was $72.2 \pm 25.4\%$, a significant increase over the inoculum. These data demonstrate that Agr-dysfunctional mutants have a significant advantage in biofilm as compared to non-biofilm infection. Thus, the development of QS mutants can be directly demonstrated in a biofilm infection model and the clinical observation of QS-dysfunctionality in chronic biofilm infection can be explained by a survival advantage QS-dysfunctional isolates have under those conditions. Notably, our results show that the development of QS dysfunctionality is specific for biofilm infection while strongly suppressed in non-biofilm infection.

We then set out to provide a mechanistic explanation for the observed survival advantage of QS-dysfunctional strains in biofilm-associated infection. The major mechanisms of innate host defense controlling the development of *S. aureus* infection are phagocytosis and killing of bacteria by leukocytes, among which neutrophils and macrophages are the most abundant. Thus, we determined whether structural differences between QS-positive and -negative biofilms impact resistance to killing by neutrophils and macrophages. Use of a live cell-specific stain revealed that QS-dependent differences of biofilm structures were even more pronounced than what our previous results using propidium iodide (PI), which stains the overall biofilm due to its interaction with extracellular DNA, had suggested¹⁴. Namely, QS-functional biofilms only consisted of sporadic microcolonies embedded in eDNA, while QS-dysfunctional or PSM-deficient biofilms formed compact layers composed mainly of living cells (Fig. 2a).

We found that the extended and compact biofilms formed by Agr- and PSM-deficient *S. aureus*¹⁴ revealed strongly decreased chances for neutrophil penetration (Fig. 2a,b). In contrast to the dense biofilms formed by the *agr* mutant, wild-type biofilms did not prevent phagocytosis (Supplementary Fig. 3). Furthermore, in higher magnification of neutrophil interaction with Agr-dysfunctional biofilms, we observed morphologies that could be interpreted as “pseudopodia” attempting to engulf bacteria, but which appeared to fail due to biofilm density (Fig. 2c). Notably, confirming the importance of these observations using a quantifiable assay, we detected significantly increased resistance to neutrophil-mediated killing by Agr- and PSM-deficient as compared to wild-type biofilms (Fig. 3). Similar

observations were made for M1 and M2 macrophages, but M2 macrophages generally had much lower capacity to eliminate bacteria than the M1 macrophages, which are known to have more pronounced bactericidal activity³⁰ (Fig. 3, Supplementary Fig. 4).

We also investigated biofilm-mediated resistance to neutrophil killing of two standard isolates belonging to pulsed-field type USA100, a major MRSA lineage associated with hospital-associated infections²⁴: N315, which is known to be Agr-dysfunctional, and 252, which was compared to an isogenic *agr* mutant. Results with the *agr*-deficient N315 and 252 *agr* were very similar to those obtained with the LAC (USA300) *agr* mutant (Fig. 2,3). Altogether, these findings serve as a mechanistic explanation of our in-vivo observations, indicating that QS dysfunctionality is subject to positive selection under conditions that are specific to the in-vivo biofilm setting.

To directly link our in-vivo findings to the observed biofilm-mediated immune evasion mechanism, we performed the biofilm-associated infection model under treatment with cyclophosphamide (CY), which destroys all leukocytes. As expected from this elimination of a major arm of innate host defense, CFU recovered from mice infected with wild-type bacteria were much higher in CY-treated as compared with non-treated mice (Fig. 4a). Importantly, the significant survival advantage that biofilms of Agr- or PSM-dysfunctional bacteria had in wild-type mice disappeared in CY-treated mice, underlining that this advantage is due to protection from leukocyte attacks. Furthermore, we observed no development of QS-dysfunctional mutants in the biofilm infection model with CY treatment, in significant contrast to the data obtained without CY treatment ($p=0.0188$, Fisher's exact test) (Tab. 1). Finally, in the biofilm infection model with 1:1 wild-type/*agr* mutant inoculation, the percentage of *agr* mutants dropped from ~ 50% to $16.5 \pm 10.0\%$ when CY was applied, a significant difference to the rise observed without CY (Fig. 4b,c). Together, these findings support the notion that the survival advantage of quorum cheaters and the development of QS dysfunctionality during biofilm infection are due to the specific structure of QS-dysfunctional biofilms that provide increased resistance to phagocyte attacks.

In conclusion, we found that the in-vivo advantage of QS dysfunctionality and the rise of QS-dysfunctional mutants (quorum cheaters) is linked specifically to biofilm-associated infection. Notably, our data mechanistically explain this infection-type dependence by the increased resistance biofilms composed of QS-dysfunctional bacteria provide to killing by phagocytes. Thus, our results indicate that QS functionality during infection is predominantly governed by the interaction of QS-controlled factors with host defenses. Furthermore, they explain the clinical observation of frequent QS mutant isolation from biofilm and device-associated blood infections^{4,5,7}. Moreover, they might explain in part why epidemiologically successful isolates of predominantly hospital-associated MRSA lineages (such as USA100) have low Agr expression or are even Agr-dysfunctional (such as N315)²³. Finally, our results support the idea that the PSMs are key contributors to the virulence and biofilm phenotypes mediated by Agr QS in *S. aureus*. Of particular interest, previous studies have suggested that quorum cheating generally occurs in bacterial infection to a considerable extent, implying severe and universal limitations to the applicability of QS blockers as therapeutics^{9,10}. In contrast, our data indicate, and mechanistically underscore,

that QS blockers are inadvisable for biofilm infection, but support the proposed therapeutic use of QS blockers in non-biofilm infection types.

Methods

Bacterial strains and culture conditions

The construction of the *psm* and *agr* isogenic mutants in the *S. aureus* USA300 LAC and 252 background strains have been described previously^{16,27,31}. For in-vitro experiments, bacteria were grown in tryptic soy broth (TSB) or TSBg (TSB with 0.5% glucose, for biofilm experiments). *S. aureus* strains were obtained from NARSA (Network on Antimicrobial Resistance in *S. aureus*: LAC, MW2, 8325–4, N315, 252) or are from a University of California San Francisco collection³² and kindly provided by Dr. B. Diep.

Analysis of QS dysfunctionality and serial passage experiments

Similar to several previous studies^{6,33}, we used hemolytic activity as a readout for Agr functionality in isolates obtained from in-vitro or in-vivo experiments. The overwhelming majority of mutants that lose hemolytic capacity reportedly show spontaneous mutations in the Agr system^{6,33}. We verified the mutation site by sequencing in the infection isolates (Supplementary Tab. 1)

For the serial passage experiments, cultures were grown in TSB for 24 h in 15-ml round-bottom tubes and fresh cultures were inoculated every day using 1/200 volume of the previous culture. On days 7, 14, 21, 35, and 42, dilutions were plated on sheep blood agar and hemolysis was evaluated after overnight incubation at 37 °C. The experiment was performed in duplicate.

Neutrophil and macrophage experiments

Human neutrophils and elutriated monocytes were obtained from healthy donors from the NIH blood bank in accordance with protocols approved by the Institutional Review Board for Human Subjects, NIAID. Informed written consent was obtained from all volunteers. Human neutrophils were isolated as previously described³⁴. Elutriated blood monocytes were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% heat-inactivated Human AB Serum (Gemini Bioproducts), 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME (all from Life Technologies) at a concentration of 1×10^6 cells per ml. To induce macrophage differentiation, C-MSF or GM-CSF (Pepro-Tech) were added to the cell cultures at a final concentration of 50 ng/ml. Medium was supplemented every 2 to 3 days. After 7 days, adhered macrophages were harvested by incubating the cells in cold PBS for 15 min on ice and gentle pipetting.

For analysis of biofilm resistance to neutrophil or macrophage killing, biofilms were grown in TSBg for 48 h in 96-well microtiter plates after inoculation using the same amount of bacteria for all strains. Biofilms were then exposed to equal numbers of neutrophils or macrophages (MOI ~ 10:1 for wild-type *S. aureus* vs. leukocytes, a factor that is somewhat higher for the *psm* and *agr* mutants due to stronger biofilm formation), and bacterial

survival was measured every 30 min up to 90 min for neutrophils and 180 min for macrophages. Investigators were blinded as for group allocation in the respective experiment.

Confocal laser scanning microscopy

Biofilms of the wild-type and isogenic *S. aureus* strains were inoculated from a pre-culture grown for 3 h in TSBg to an OD₆₀₀ of 0.1 in a Nunc Lab-Tek II Chamber Slide System, and grown at 37 °C for 48 h in the same media. They were then stained first with PI (15 min) and then with SYTO 9 (50 nM, 30 min). Human neutrophils or macrophages were isolated as above and stained with 20 µM CellTrace Violet Cell Proliferation Dye (C34557, Life Technology). Stained neutrophils or macrophages were added to grown biofilms and imaged after 30 minutes (neutrophils) or 2 h (macrophages) of incubation at 37 °C with a Zeiss LSM 700 Laser Scanning Microscope using three confocal channels (405 nm laser for the neutrophils or macrophages stained in violet, 555 nm laser for the PI-stained staphylococcal biofilm, and 488 nm for SYTO 9).

Confocal images were analyzed using the Zen 2010B SP1 software [version 6.00.485] and saved as .lsm files. These .lsm files were converted into .ims (IMARIS) files for biofilm statistical analyses and image production in IMARIS. Total and average biovolumes were calculated in IMARIS version 9.0.0. Mean thickness was calculated using the Biofilm Analysis XTension (Bitplane) in IMARIS version 9.0.0. XTension was written for Imaris 8.2.0 using Matlab 2015a. Penetration of neutrophils into the biofilm layer was quantified manually by subtracting the bottom Z layer value of the green (SYTO 9) channel from the bottom Z layer values for each of the cells in the yellow (neutrophil) channel. These values show how far the bottom of each cell has penetrated into the biofilm.

Agr sequencing

The *agr* system was sequenced in its entirety using the oligonucleotides listed in Supplementary Table 2 in all non-hemolytic isolates obtained in the animal experiments. Supplementary Table 1 lists the location of mutations.

Animal experiments

All mouse experiments were performed at the animal care facilities of the NIAID in accordance with ethical guidelines approved by the Animal Care and Use Committee of the NIAID. All animals were euthanized by CO₂ at the end of the studies. C57BL/6J mice were purchased from Jackson. All experiments were performed using age-matched females, between 6 to 10 weeks old. No specific statistical consideration was taken in determining the sample size for the animal experiments.

The mouse skin infection model was performed essentially as previously described¹⁶. Briefly, $\sim 1 \times 10^7$ CFU of bacteria in 50 µl of PBS were injected subcutaneously into the left and/or right flank of shaved mice. For the 1:1 (wild-type/ *agr*) mixed infection model, $\sim 5 \times 10^6$ CFU of each strain were injected together in 100 µl of PBS. Total abscess sizes were measured at 24-h intervals for a total of 6 days post infection using an electronic caliper, as described²⁷. Open lesion sizes were determined at 24-h intervals using an IVIS Lumina XR

imager (Perkin Elkins) with the following settings; field of view 12.5, F stop 16, and an exposure time of 0.2 seconds. Images were loaded into ImageJ (version 1.5) and the abscess areas (mm²) were measured using a scale of 5.5 pixels/mm. To determine the bacterial load, abscesses on day 6 were surgically removed, cut into small pieces, and split into four separate tubes containing 500 µg of borosilicate glass beads and 500 µl of sterile PBS. The skin pieces were homogenized in a FastPrep 96 (MP Bio) homogenizer, 5 × 1 min at 1800 rpm. The homogenates were then plated, incubated at 37 °C for 24 h, and enumerated. Proportions of wild-type versus *agr* bacteria were determined by plating on tryptic soy agar (TSA), where both grow, and TSA with 3 µg/ml tetracycline, where only *agr* bacteria grow due to the tetracycline resistance gene introduced to replace the *agr* locus.

The catheter infection model was performed essentially as described previously³⁵. Briefly, 1-cm catheter pieces were coated with equal numbers of bacteria. Adherence was tested in control experiments and was in the same range (~ 10³ - 10⁴), with wild-type generally adhering better than *agr* or *psm* bacteria. For the 1:1 (wild-type/ *agr*) mixed infection model, adherence of wild-type and mutant bacteria was confirmed on five control catheters to be equal (*agr*, 43.4 ± 8.4%). Catheters were then inserted under the dorsal skin of mice. On day 6, catheters were removed and CFU on the catheters and the directly adjacent tissue were determined by plating as above, after performing a validated sonication protocol to disrupt bacterial agglomerates on the catheters, and homogenizing the tissue samples using the FastPrep 96 (MP Bio) homogenizer, 1 × 1 min.

For treatment with cyclophosphamide monohydrate (CY)³⁶, CY (Sigma-Aldrich) was dissolved in water to a concentration of 1.5 mg/ml or 1 mg/ml and filter-sterilized. Seven days prior to challenge, mice were given an intraperitoneal injection at a dosage of 150 mg/kg of body weight with 2 ml of 1.5 mg/ml CY. Then mice were treated every 48 h via intraperitoneal injection with CY (1 mg/ml) dissolved in 2 ml of sterile water at a dose of 100 mg/kg body weight for three times prior to the infection.

Investigators were blinded as for group allocation when determining CFU in the experiment shown in Fig. 1 and the corresponding Supplementary Fig. 2.

Statistics

Statistical analysis was performed using Graph Pad Prism version 7.0. All error bars depict the standard deviation (SD). Horizontal lines depict the mean. All replicates are biological (from different samples). Tukey or Dunnett post-tests for ANOVA correct for multiple comparisons using statistical hypothesis testing.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files) or available from the corresponding author upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH) (project number 1 ZIA AI000904, to M.O.), The National Natural Science Foundation of China (grant 81501804, to L.H.), and the General Program of Shanghai Municipal Commission of Health and Family Planning (grant 20154Y0014, to L.H.).

References

1. Asfahl KL & Schuster M Social interactions in bacterial cell-cell signaling. *FEMS Microbiol Rev* 41, 92–107, doi:10.1093/femsre/fuw038 (2017). [PubMed: 27677972]
2. Miller MB & Bassler BL Quorum sensing in bacteria. *Annu Rev Microbiol* 55, 165–199, doi:10.1146/annurev.micro.55.1.165 (2001). [PubMed: 11544353]
3. Dandekar AA, Chugani S & Greenberg EP Bacterial quorum sensing and metabolic incentives to cooperate. *Science* 338, 264–266, doi:10.1126/science.1227289 (2012). [PubMed: 23066081]
4. Bjarnsholt T et al. Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. *PLoS One* 5, e10115, doi:10.1371/journal.pone.0010115 (2010). [PubMed: 20404933]
5. Vuong C, Kocianova S, Yao Y, Carmody AB & Otto M Increased colonization of indwelling medical devices by quorum-sensing mutants of *Staphylococcus epidermidis* in vivo. *J Infect Dis* 190, 1498–1505, doi:10.1086/424487 (2004). [PubMed: 15378444]
6. Traber KE et al. agr function in clinical *Staphylococcus aureus* isolates. *Microbiology* 154, 2265–2274, doi:10.1099/mic.0.2007/011874-0 (2008). [PubMed: 18667559]
7. Fowler VG Jr. et al. Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. *J Infect Dis* 190, 1140–1149, doi:10.1086/423145 (2004). [PubMed: 15319865]
8. Dickey SW, Cheung GYC & Otto M Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. *Nat Rev Drug Discov* 16, 457–471, doi:10.1038/nrd.2017.23 (2017). [PubMed: 28337021]
9. Rumbaugh KP et al. Quorum sensing and the social evolution of bacterial virulence. *Curr Biol* 19, 341–345, doi:10.1016/j.cub.2009.01.050 (2009). [PubMed: 19230668]
10. Pollitt EJ, West SA, Crusz SA, Burton-Chellew MN & Diggle SP Cooperation, quorum sensing, and evolution of virulence in *Staphylococcus aureus*. *Infect Immun* 82, 1045–1051, doi:10.1128/IAI.01216-13 (2014). [PubMed: 24343650]
11. Gordon RJ & Lowy FD Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* 46 Suppl 5, S350–359, doi:10.1086/533591 (2008). [PubMed: 18462090]
12. Le KY & Otto M Quorum-sensing regulation in staphylococci-an overview. *Front Microbiol* 6, 1174, doi:10.3389/fmicb.2015.01174 (2015). [PubMed: 26579084]
13. Otto M Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. *Annu Rev Med* 64, 175–188, doi:10.1146/annurev-med-042711-140023 (2013). [PubMed: 22906361]
14. Periasamy S et al. How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A* 109, 1281–1286, doi:10.1073/pnas.1115006109 (2012). [PubMed: 22232686]
15. Peschel A & Otto M Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol* 11, 667–673, doi:10.1038/nrmicro3110 (2013). [PubMed: 24018382]
16. Wang R et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 13, 1510–1514, doi:10.1038/nm1656 (2007). [PubMed: 17994102]
17. Painter KL, Krishna A, Wigneshweraraj S & Edwards AM What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol* 22, 676–685, doi:10.1016/j.tim.2014.09.002 (2014). [PubMed: 25300477]

18. Collins J et al. Offsetting virulence and antibiotic resistance costs by MRSA. *ISME J* 4, 577–584, doi:10.1038/ismej.2009.151 (2010). [PubMed: 20072161]
19. Paulander W et al. Antibiotic-mediated selection of quorum-sensing-negative *Staphylococcus aureus*. *MBio* 3, e00459–00412, doi:10.1128/mBio.00459-12 (2013). [PubMed: 23143800]
20. Vuong C, Saenz HL, Gotz F & Otto M Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* 182, 1688–1693, doi:10.1086/317606 (2000). [PubMed: 11069241]
21. Shopsis B et al. Mutations in *agr* do not persist in natural populations of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 202, 1593–1599, doi:10.1086/656915 (2010). [PubMed: 20942648]
22. Vuong C, Kocianova S, Yu J, Kadurugamuwa JL & Otto M Development of real-time in vivo imaging of device-related *Staphylococcus epidermidis* infection in mice and influence of animal immune status on susceptibility to infection. *J Infect Dis* 198, 258–261, doi:10.1086/589307 (2008). [PubMed: 18491976]
23. Li M et al. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* 106, 5883–5888, doi:10.1073/pnas.0900743106 (2009). [PubMed: 19293374]
24. Carrel M, Perencevich EN & David MZ USA300 Methicillin-Resistant *Staphylococcus aureus*, United States, 2000–2013. *Emerg Infect Dis* 21, 1973–1980, doi:10.3201/eid2111.150452 (2015). [PubMed: 26484389]
25. Kourbatova EV et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA 300 clone as a cause of health care-associated infections among patients with prosthetic joint infections. *Am J Infect Control* 33, 385–391, doi:10.1016/j.ajic.2005.06.006 (2005). [PubMed: 16153484]
26. Foster TJ Immune evasion by staphylococci. *Nat Rev Microbiol* 3, 948–958, doi:10.1038/nrmicro1289 (2005). [PubMed: 16322743]
27. Cheung GY, Wang R, Khan BA, Sturdevant DE & Otto M Role of the accessory gene regulator *agr* in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *Infect Immun* 79, 1927–1935, doi:10.1128/IAI.00046-11 (2011). [PubMed: 21402769]
28. Butterfield JM et al. Predictors of *agr* dysfunction in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates among patients with MRSA bloodstream infections. *Antimicrob Agents Chemother* 55, 5433–5437, doi:10.1128/AAC.00407-11 (2011). [PubMed: 21930887]
29. Nowakowska J, Landmann R & Khanna N Foreign Body Infection Models to Study Host-Pathogen Response and Antimicrobial Tolerance of Bacterial Biofilm. *Antibiotics (Basel)* 3, 378–397, doi:10.3390/antibiotics3030378 (2014). [PubMed: 27025752]
30. Flannagan RS, Heit B & Heinrichs DE Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of *Staphylococcus aureus*. *Pathogens* 4, 826–868, doi:10.3390/pathogens4040826 (2015). [PubMed: 26633519]
31. Joo HS, Cheung GY & Otto M Antimicrobial activity of community-associated methicillin-resistant *Staphylococcus aureus* is caused by phenol-soluble modulins derivatives. *J Biol Chem* 286, 8933–8940, doi:10.1074/jbc.M111.221382 (2011). [PubMed: 21278255]
32. Diep BA, Carleton HA, Chang RF, Sensabaugh GF & Perdreau-Remington F Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 193, 1495–1503, doi:10.1086/503777 (2006). [PubMed: 16652276]
33. Somerville GA et al. In vitro serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and *agr* nucleotide sequence. *J Bacteriol* 184, 1430–1437 (2002). [PubMed: 11844774]
34. Voyich JM et al. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J Infect Dis* 194, 1761–1770, doi:10.1086/509506 (2006). [PubMed: 17109350]
35. Wang R et al. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. *J Clin Invest* 121, 238–248, doi:10.1172/JCI42520 (2011). [PubMed: 21135501]

36. Cox PJ, Phillips BJ & Thomas P The enzymatic basis of the selective action of cyclophosphamide. *Cancer Res* 35, 3755–3761 (1975). [PubMed: 172233]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. QS in biofilm- and non-biofilm-associated infection.

a, Non-biofilm (skin abscess) model. Mice were subcutaneously injected with 1×10^7 CFU and CFU in the abscesses were determined at day 6 post infection. WT, wild-type USA300 LAC (n=10); *agr*, *psm*, isogenic *agr* and *psm* deletion mutants (n=8 each). See Supplementary Fig. 2 for abscess and open lesion sizes over the course of the experiment. **b**, Biofilm (catheter) model. 1-cm catheter pieces coated with approximately equal numbers of bacteria (between 10^3 and 10^4 CFU per catheter piece) were inserted under the dorsum of mice (2 per mouse; n=10 mice per strain). CFU on the catheter and directly surrounding tissue were determined at day 6 post infection. Statistical analysis in the experiments shown in panels **a** and **b** is by 1-way ANOVA with Dunnett's post-test versus data obtained with WT. **c-e**, The device-related biofilm and the non-biofilm skin abscess model were performed

using ~ 1:1 mixed inocula of wild-type and *agr* (QS-deficient) bacteria. In both models, CFU were determined in the abscesses or on the catheters, respectively, 6 days after injection. **c**, Skin infection model. The model was performed with n = 10 mice. The inoculum contained 5×10^6 CFU each of WT and *agr* bacteria. **d**, Catheter infection model. The model was performed with n = 5 mice and 2 catheters implanted per mouse on the left and right dorsum. One catheter fell out during the model and was not counted (total n=9). Statistical analysis in the experiments shown in panels **c** and **d** is by two-tailed paired t-tests. **e**, Data achieved per abscess or catheter in panels **c** and **d** expressed as percentage of *agr* among total bacteria. Bacteria attached *in vitro* to control catheters (“catheter inoculum”) were measured to estimate the inoculated bacteria (n=5). Statistical analysis is by two-tailed unpaired t-tests (skin infection versus catheter infection; catheter infection versus control catheters.) **a-e**, Error bars show the mean $\pm$ SD.

Fig. 2. QS-dysfunctional *S. aureus* forms compact biofilms that prevent neutrophil penetration.

a, Confocal laser scanning microscopy (CLSM) images of biofilms [stained with PI (red), which stains extracellular DNA, and SYTO 9 (green) staining live cells] formed by the indicated *S. aureus*, with and without application of neutrophils (yellow). Biofilms were grown for 48 h and neutrophils were added at equal numbers. The top rows show biofilm structures without neutrophils, and the other three rows with neutrophils from two different angles (“diamond” view from above and side view), as well as with only SYTO 9 staining in side view. CLSM experiments were repeated at least once giving similar results. **b**, Neutrophil penetration into the biofilm, based on SYTO 9-only stain. The total number of neutrophils for every strain in the shown field of view was analyzed [$n=24$ (LAC), $n=13$ (LAC Δagr), $n=8$ (LAC Δpsm), $n=12$ (252), $n=8$ (252 Δagr), $n=16$ (N315)]. Statistical analysis is by 1-way ANOVA with Dunnett’s post-test versus WT (for LAC series), and by unpaired two-tailed t-tests for 252 versus 252 Δagr . *, $P<0.0001$. Error bars show the mean $\pm$ SD. **c**, Example CLSM side-view images in higher magnification. Note structures that can be interpreted as pseudopodia attempting to (unsuccessfully) engulf bacteria in the QS-deficient mutant biofilm. See Supplementary Fig. 4 for pictures of the corresponding experiments performed with human macrophages.

Fig. 3. QS-dysfunctional biofilms prevent killing by leukocytes.

Quantitative analysis of survival of biofilms formed by the same strains as in Fig. 2 upon incubation with neutrophils, and biofilms formed by LAC wild-type and its isogenic *agr* and *psm* mutants upon incubation with M1 or M2 macrophages. Biofilms were grown in TSBg for 48 h and the leukocytes were added at equal numbers. Experiments were performed in triplicate (n=3). Statistical analysis in the graphs showing experiments with LAC is by 2-way ANOVA with Tukey's multiple comparison post-test; shown comparisons are versus WT. Values obtained with 252 and 252 *agr* are compared by two-tailed unpaired t-tests at every time point. Values are shown as percentages as compared to the value at t=0 min for every strain, which was set to 100%. Error bars show the mean $\pm$ SD.

Fig. 4. Benefits of QS mutants in biofilm-associated infection are due to interaction with leukocytes.

The biofilm (catheter) model was performed with cyclophosphamide (CY), which destroys leukocytes, to determine to which extent the achieved in-vivo results are due to biofilm-mediated resistance to leukocytes. Mice received an initial CY dose of 150 mg/kg body weight and three further doses of 100 mg/kg body weight in 48-h intervals before inoculation of the bacteria the day after the last dose. Results from Fig. 1 are included for direct comparison in panels **a-c**. All experimental details are the same as in Fig. 1. **a**, Catheter model (separate infections). Statistical analysis is by 1-way ANOVA with Dunnett's comparison post-test versus data obtained with WT. $n=10$ mice with 2 catheters each. **b**, Catheter model (1:1 WT/ Δagr infection; $n=9$). Statistical analysis is by two-tailed paired t-tests. **c**, Data achieved in the experiment shown in panel **b** (and the corresponding

experiment without CY treatment from Fig. 1) expressed as percentage of *agr* among total bacteria. Statistical analysis is by two-tailed unpaired t-test. **a-c**, Error bars show the mean $\pm$ SD.

Tab. 1

Rates of QS-dysfunctional mutant development in biofilm- and non-biofilm infection

Mice/ condition	Catheter biofilm infection QS mutant rate ¹	Skin abscess infection QS mutant rate ¹
untreated	5×10^{-3} (5/1014) ²	0 (0/1209; p=0.0196) ³
CY ⁴ -treated	0 (0/1228; p=0.0188) ³	N.D. ⁵

¹Frequency (non-hemolytic among total isolates tested). At least 100 clones each were analyzed from 10 catheters (catheter biofilm infection), or from n=10 abscess samples (skin abscess infection).

²See Supplementary Table 1 for *agr* mutations in the 5 non-hemolytic clones obtained from WT-infected mice.

³Fisher's exact test.

⁴CY, cyclophosphamide

⁵N.D., not determined.