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Confinement-Induced Quorum Sensing of Individual *Staphylococcus aureus* Bacteria

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Abstract

It is postulated that, in addition to cell density, other factors, such as the dimensions and diffusional characteristics of the environment, could influence quorum sensing (QS) and induction of genetic reprogramming. Modeling studies predict that QS may operate at the level of a single cell, but, due to experimental challenges, the potential benefits of QS by individual cells remain virtually unexplored. Here we report a physical system that mimics isolation of a bacterium, such as within an endosome or phagosome during infection, and maintains cell viability under conditions of complete chemical and physical isolation. For *Staphylococcus aureus*, we show quorum sensing and genetic re-programming to occur in a single isolated organism. Quorum sensing allows *S. aureus* to sense confinement and to activate virulence and metabolic pathways needed for survival. To demonstrate the benefit of confinement-induced quorum sensing to individuals, we showed quorum sensing bacteria to have significantly greater viability over non-QS bacteria.

Some bacteria, including medically relevant pathogenic bacteria produce, secrete, and sense small, hormone-like signaling molecules, termed autoinducers, whose extracellular concentrations regulate gene expression and control multiple important functions including virulence and biofilm formation^{1,2}. The prevailing view is that this signaling allows populations of cells to assess their density i.e. to quorum sense. The QS hypothesis is that, because the local autoinducer concentration can be cell density dependent, bacteria use signaling to monitor the environment for other like bacteria. When a quorum is detected, genetic reprogramming occurs to coordinate cooperative behaviors at the population level, providing group benefits that would be unproductive at lower density³. However cells are unable to distinguish between cell density and other factors influencing extracellular autoinducer concentration such as mass transport, confinement, and degradation, since a response is triggered only if the rates of autoinducer production, mass transfer and decay

integrated over time reach a threshold concentration at the cell's location⁴. Thus we expect that, in addition to cell density, other physical and chemical factors, such as the dimensions and diffusional characteristics of the environment, could influence induction of genetic reprogramming^{5,6}. On this basis Redfield⁷ proposed QS to be diffusion sensing – the ability to determine whether secreted molecules rapidly move away from the cell, thereby allowing regulation of secretion of degradative enzymes and effectors to minimize losses due to diffusion or convection. Recently, Hense et al.⁴ proposed the concept of efficiency sensing (ES): cells sense a combination of cell density, mass-transfer properties, and spatial cell distribution to estimate the efficiency of producing extracellular effectors and to respond only when this is efficient. These alternative QS motives depend strictly on local autoinducer concentration and should operate at the individual organism level. This is important, because complex behavior needs to be invoked to account for both QS evolution and maintenance in groups, as 'cheating' can occur, where individuals exploit secreted group resources without contributing equally to their generation^{3,8}. To reconcile these different perspectives, we hypothesized that QS, independent of its recognized group benefits, could operate also at the single cell level to provide fitness benefits to individual bacteria that could be selected for. To test this hypothesis, we developed a physical system to isolate individual *S. aureus* and examined confinement-induced effects on signaling, gene expression, and viability. Here we show self-induction and resultant genetic reprogramming to occur efficiently in isolated individual organisms, enabling adaptation and survival. It is noteworthy that, while there are data that suggest small numbers of intracellular *S. aureus* can undergo QS⁹ and recent modeling suggests that as few as two cells could induce QS^{10,11}, the ability of a single bacterium to quorum sense in a confined space has not been tested definitively. In fact no study has ever been performed of any physiologic process in bacteria that could examine unambiguously the behavior of a single bacterium in a confined space.

In *S. aureus*, the accessory gene regulator *agr* operon is responsible for QS regulation². It contains two divergent transcripts, RNAII and RNAPIII driven by activation of two promoters, P2 and P3, respectively. RNAII encodes four genes, *agrBDCA*, that are required to synthesize, export, and detect an autoinducing cyclic peptide, AIP. AgrC and AgrA form a two-component regulatory pair. AIP binding to its surface receptor, AgrC, activates a phosphorylation cascade inducing expression of RNAPIII, a regulatory RNA that represses adhesin expression and up-regulates an array of toxins, hemolysins, degradatory enzymes, and metabolic pathways. Micro-array studies have revealed that 104 genes are up-regulated and 34 are down-regulated as a result of QS, representing ~5% of the genome¹². AgrA-P also induces expression of the RNAII transcript, exerting positive feedback control on this regulatory system¹³.

RESULTS

Physical and chemical Isolation of *S. aureus* in a nanostructured matrix

To date, quorum sensing in *S. aureus* has been studied with large numbers of bacteria ($1 \times 10^7 - 1 \times 10^9$) in either broth suspension cultures or cell cultures of phagocytosed bacteria¹⁴. Therefore, the potential for individual staphylococci to autoinduce in the absence

of neighboring bacteria or cell signaling interference inherent to these systems (e.g. through interactions with the phagocytosing host cells) is currently unknown. To observe QS in isolated, individual cells, *S. aureus* were immobilized, individually (or in small groups, see Supplementary Fig. 1 online), within a matrix fabricated at a sufficiently small physical scale ($\sim 20\ \mu\text{m}$ diameter, physically isolated hemispherical droplets, see Fig. 1A and B) so that the overall cell density (~ 1 cell per $2 \times 10^3\ \mu\text{m}^3$, equivalent to $\sim 0.5 \times 10^9$ cells mL^{-1}) exceeded the reported QS threshold ($10^8 - 10^9$ cells mL^{-1})¹⁵. The matrix was formed by adaptation of our cell-directed assembly approach¹⁶ to an aerosol procedure we developed previously to form ordered porous silica nanospheres¹⁷. It results in cells incorporated within a dihexanoylphosphatidylcholine (*diC₆PC*) lipid vesicle (Fig. 1C) maintained at a pH of ~ 5.5 (Fig. 1D), approximating that of the early endosome, pH 5.4–6.2, depending on cell type¹⁸, and surrounded by an ordered silicon dioxide nanostructure (Fig. 1A and B) that serves as a reservoir for any added buffer and media. This construct mimics some of the physical and chemical features of a bacterium entrapped within an intracellular membrane-bound compartment (endosome or phagosome), although we note that the shorter chain *diC₆PC* lipid bilayers are expected to be somewhat more permeable than their longer chain counterparts, and that the chemical environment of an endosome or phagosome is more complex than we can achieve in our reduced system. The concentration of the bacterium isolated in a vesicle is approximately 0.5×10^{12} cells mL^{-1} . As discussed below, it is the smaller volume of the vesicle that establishes the effective cell density ($\gg$ QS threshold) and the relevant volume in which AIP can accumulate to trigger a response. Importantly, this architecture, *viz* a vesicle-enveloped cell incorporated in a much larger nanostructured silica bead (Fig. 1A and B), allows individual cells to be maintained in a viable state under externally dry conditions¹⁹ that establish complete physical and chemical isolation of one cell from all others. This reduced physical system is biologically relevant, because *Staphylococcus aureus* is known to become trapped in such intracellular compartments²⁰, and it is proposed that they employ a QS strategy to induce new gene expression, promoting intracellular survival and/or escape^{14,15,20}. However it is presently unknown whether confinement alone can promote QS or whether other factors within the endosomal organelle are required. We use our system to test confinement alone as a mechanism for inducing QS.

Monitoring induction of quorum sensing pathways in isolated individual *S. aureus*

To optically monitor the onset and kinetics of auto-induced QS, we used *S. aureus* strains ALC1743 (*agr* group 1 RN6390 containing reporter *agr*: P3-gfp) and ALC1740 (RN6390 containing reporter *hla*-gfp) at an early exponential phase prior to QS induction. Expression of green fluorescent protein (GFP) by ALC1743 reports quorum sensing-dependent *agr* P3-promoter activation (as it would occur in the late exponential phase of growth in broth culture), while in ALC1740 it reports QS-mediated downstream synthesis of the pore-forming toxin, α -hemolysin. As a negative control we used strain ALC6513 (an *agrA*(–) mutant containing reporter *agr*: P3-gfp) – because this strain uses the exact same reporter construct as ALC1743 but lacks AgrA, one component of the two component regulatory pair, it tests for the possibility of non-AIP induced GFP expression. Figure 2A–B show representative confocal images of isolated, red-stained ALC1743 immediately following encapsulation and after ten hours of incubation at 37° C. As seen in the accompanying kinetic plot (Fig. 3A), GFP expression follows a sigmoidal curve. It initiates over one hour

and increases progressively with time to over 90% at ten hours where it begins to level off. (Equivalent QS activation was also obtained for the Newman strain (a clinical isolate) containing reporter *agr.P3-gfp* (see Supplementary Fig. 2 online), confirming that our observations were not unique to the laboratory strain RN6390 that has a genetic alteration that makes it different from some clinical isolates). The time course of GFP expression in isolated cells is qualitatively similar to but slightly more accelerated than that of the same strains maintained in broth culture at concentrations exceeding the QS threshold²¹. This is presumably a consequence of localized confinement and restricted transport of extracellular AIP in our nanostructured system compared to that in broth cultures. Over the 24 hour time course we observed no measurable GFP expression from strain ALC6513.

Confinement-induced quorum sensing pathways in isolated individual *S. aureus* are sensitive to exogenous inducers and inhibitors

Figure 3B depicts the time course for GFP expression of ALC1743 isolated in droplets to which exogenous type 1 AIP or the QS inhibitor, very low density lipoprotein (VLDL)²², was added immediately prior to the aerosol assembly process. We observe cyclic AIP1 to accelerate significantly GFP expression relative to the corresponding ALC1743 sample prepared without exogenous AIP. In contrast VLDL suppresses GFP expression for 10 hours, after which expression kinetics paralleling those of ALC1743 are recovered. As recently reported²², the mechanism of VLDL inhibition of quorum sensing in *S. aureus* involves binding of the major structural protein of this lipoprotein, apolipoprotein B, to AIP1 preventing binding to the AgrC receptor and antagonizing the QS signaling cascade. For confined cells, GFP expression presumably commences once the local extracellular AIP concentration increases through cellular production and export to become comparable to that of extracellular VLDL. Fig. 3b also plots GFP expression for the *agrA*(-) mutant strain isolated for 24 hours and then dosed with exogenous AIP1. No GFP expression was observed for times up to 24 hours. For all sets of data we observe an insignificant effect of small groups (2–8 cells) versus individuals on expression kinetics (Chi square analysis). Collectively the data in Figs. 3A and B, showing sensitivity to inducers and inhibitors and no GFP expression for the negative control, indicate that our physical system monitors QS induced by the *agr* encoded two-component regulatory system, as opposed to other possible confinement-induced outcomes. The similar behavior of individuals and groups suggests that the exogenous AIP or VLDL is incorporated within the vesicles, which surround and isolate cells incorporated individually or in groups, and that it is the vesicle compartment that establishes the volume and effective localized, integrated concentration of cell-secreted plus exogenous AIP activator or inhibitor responsible for regulation of gene expression.

Confinement induced quorum sensing of isolated individual *S. aureus* up-regulates expression of virulence factors

Figure 2C and D show representative confocal images of isolated, individual *S. aureus* strain ALC1740 and Fig. 3A shows the corresponding time course of GFP expression. The progressively increasing GFP expression over 10 hours mirrors that of QS (Fig. 3A) and shows activation of the RNAPII-dependent pathway that induces expression of secreted virulence factors. Here we specifically detect activation of the α -hemolysin promoter. Although there are data that suggest that small numbers of intracellular *S. aureus* quorum

sense²⁰, the combined data in Figures 2 and 3 provide the first proof of auto-induction of an individual, physically and chemically isolated organism. Additionally these data provide the first evaluation of gene expression kinetics for a large population of isolated individual cells. We postulate that quorum sensing allows isolated *S. aureus* to sense confinement through increased extracellular concentration of autoinducer and to activate virulence factor pathways and initiate new gene expression needed to survive in such confined environments¹⁵. For both QS and α -hemolysin expression we see no statistical difference between isolated individuals and small groups. This enforces the supposition that our assembly process incorporates cells (individuals or groups) in vesicle compartments that establish the localized extracellular AIP concentration that triggers QS and expression of secreted factors.

Confinement-induced quorum sensing enhances survival of isolated individual *S. aureus*

To demonstrate the benefit of discrete quorum sensing to individuals, we compared the viability of isolated, individual RN6390 to that of RN6911, a RN6390 mutant unable to initiate QS due to deletion of the *agr* operon. RN6390 and RN6911 were isolated in nanostructured lipid/silica droplets prepared with or without incorporation of nutrient (media) in the nanostructured host matrix. Figure 3C shows that, over an 18-day incubation period confined within the media-containing nanostructured lipid/silica droplet at 37° C, the viability of RN6390 (*agr*+) was significantly greater than that of the isolated mutant RN6911 (*agr*-) ($P = 0.046$, compare Fig. 3C, Plots 1 and 2). A plausible explanation for the viability difference is that confinement-induced QS and attendant up-regulation of a spectrum of genes affecting virulence and metabolism enhances utilization of external nutrients. Consistent with this idea, the viability of *agr*+ isolated in comparable nanostructured lipid/silica droplets self-assembled without nutrients (Fig. 3C, Plot 3) was statistically equivalent to that of the *agr*- mutant isolated in a nutrient containing matrix (Fig. 3C, Plot 2) and that of the additional control, *agr*-, immobilized within a matrix fabricated without nutrients (Fig. 3, Plot 4). These data support the idea that QS poises isolated cells to access and utilize nutrients. The plausibility of this argument is further supported by control experiments with *agr*+ and *agr*- performed in broth cultures. Supplementary Figure 3 online shows that, after 47 hours of incubation at 37° C, cells able to quorum sense have significantly greater viability. It is noteworthy that these experiments, prompted by behavior in our reduced system, are the first to show this *agr*+ advantage, which is observed for both laboratory strains and recent clinical isolates like MRSA USA300 (data not shown).

DISCUSSION

By use of a reduced physical system, devoid of inter-cellular signaling interference inherent to bulk cultures^{23,24} and previous studies of endosomal entrapment^{9,20} we demonstrated confinement-induced quorum sensing for an individual isolated organism. *S. aureus* entrapped individually within a small volume senses and responds to confinement through accumulation of extracellular AIP and activation of the two-component response regulatory system with its inherent positive-feedback control. We propose that up-regulation of the *agr* effector molecule RNAPIII enhances the expression of a diverse array of genes associated

with metabolism, transport, and virulence^{12,15}. As implied by our viability studies, one benefit derived by autoinduction is the poisoning of isolated cells to be able to scavenge for and utilize external nutrients and thus better survive in isolation. Perhaps more important are the overall implications for bacterial pathogenicity. Unlike in batch cultures, bacteria, certainly pathogens, are often found in small numbers (e.g. in the gut or respiratory track) and in enclosed spaces. Our results imply that, shortly after colonization, individual or small groups of cells initiate virulence factor expression. Therapies aimed at inhibiting quorum sensing are therefore promising strategies for eradication of infection at its outset^{22,25}.

Concerning taxonomy of QS, the confinement-induced QS we report is consistent with the fully articulated QS model and its inherent sensitivity to external factors such as the dimensions and diffusional characteristics of the environment⁵. However, it is important to re-emphasize that induction of genetic reprogramming depends on autoinducer concentration exceeding a threshold value at the cell surface, and cells cannot distinguish between the three key determinants of autoinducer concentration, *viz.* cell density, mass-transfer properties, and spatial distribution of cells⁴. Our results clearly illustrate that under certain conditions induction can be independent of both cellular density and spatial distribution. Thus the term quorum sensing, and its implicit definition of ‘sensing a quorum’, is a misnomer, especially when applied to isolated, individual cells. Furthermore our results confirm one experimental prediction of the diffusion-sensing hypothesis posed by Redfield, “that isolated cells should be able to produce enough autoinducer for self-induction under plausible natural conditions”⁷ But as to whether autoinducer peptide controlled genetic-reprogramming should be classified as QS¹, DS⁷, or ES⁴, we advocate a systems biology perspective where the underlying two-component regulatory system is inherently sensitive to the combined factors that control the concentration of extracellular autoinducer peptides proximate to the cell surface. This view readily extends the QS concept and attendant benefits to the individual cell level, where it is unnecessary to invoke complex social interactions for its evolution and maintenance. Importantly, it emphasizes that for medically important pathogens such as *S. aureus*, QS can contribute significantly to the survival of the isolated individual²⁶ as we showed in our reduced physical system.

METHODS

Cell lines used

The *Staphylococcus aureus* strains used in this study, ALC1743 (*agr* group 1 RN6390 containing reporter *agr*: P3-gfp), ALC1740 (RN6390 containing reporter *hla*-gfp), ALC1743 (RN6390 *agr* deletion mutant containing reporter *agr*: P3-gfp), wild-type Newman containing reporter *agr*.P3-gfp, wild-type RN6390, and RN6911 (RN6390 *agr* deletion mutant) were generated and grown in trypticase soy broth (TSB, from Becton, Dickinson and Company) to early exponential phase before freezing in stock^{27,28}.

Preparation of nanostructured silica droplets containing live cells

Isolated nanostructured droplets containing individual cells (or small groups of cells, see e.g. Supplementary Fig. 1 online) were prepared by an extension of our evaporation-induced self-assembly process where an amphiphilic short chain phospholipid was used as a

biocompatible structure directing agent^{16,17,19,29}. Upon evaporation, lipids direct the organization of silica into an ordered lipid/silica nanostructure, which serves in our experiment as a synthetic intracellular milieu in which to incorporate individual cells. To prepare these droplets, stock solutions of soluble silica precursors were prepared by refluxing tetraethylorthosilicate (TEOS, from Sigma), ethanol, de-ionized water and HCl (molar ratios 1: 4: 1: 5×10^{-5}) for 90 minutes at 60°C. Water, HCl and TSB (a media serving as a nutrient required for GFP expression) were added to the stock solution to achieve a biologically compatible sol with final molar ratios of 1 TEOS: 4 ethanol: 0.01 HCl: 6 water: 6 TSB. 30 mg/mL of the C6 phospholipid, dihexanoylphosphatidylcholine (from Avanti Polar Lipids), was then added to the silica solution along with any exogenous materials - cyclic AIP1 (from Commonwealth, Inc. in Richmond, VA) at a concentration of 100 nM, exceeding the threshold for induction of QS through exogenous cyclic AIP1 addition¹³, or VLDL (from US Biological) at $10 \mu\text{g mL}^{-1}$. Stocks of the various cell strains (not previously expressing GFP) were centrifuged and immediately resuspended/diluted in water. These cells were added to the silica/lipid solution to yield a final concentration of 10^6 cells $\text{mL}^{-1} \ll$ quorum sensing threshold. The sol was immediately aerosol deposited onto glass resulting in physically and chemically isolated (approximately) hemispherical droplets (Supplementary Fig. 1, A) containing individual or small groups of cells as determined by confocal microscopy (see below). The silica matrix is characterized by a periodic uniform lipid/silica nanostructure as confirmed by small angle x-ray scattering (Supplementary Fig. 1, B). Encapsulated cells prepared with either optically labeled lipid (NBD, from Avanti Polar Lipids) or a fluorescent pH probe (Oregon Green, from Invitrogen) allowed visualization of lipid localization around the cell or maintenance of a localized physiologically buffered pH (Supplementary Fig. 1, I, J), similar to that reported previously for our cell-directed assembly process¹⁶. Individual cell-containing droplets are maintained in air and separated by air gaps with spacings comparable to or exceeding the droplet diameters (10–20 μm), preventing any AIP diffusion between droplets during experiments.

Imaging and determination of GFP induction

Following deposition, droplets were incubated at 37°C for indicated periods of time in air (nanostructure maintains and supplies water and nutrients). No growth or division of the isolated cells was readily observed. Additional samples were also refrigerated for identical periods of time and used to verify the absence of GFP expression in cell stocks. After incubation, samples were stained with 50 μM SYTO 64 for 45 minutes at 37°C for visualization, washed 3 times with DI water, fixed with 4% formaldehyde for 45 minutes, again washed 3 times with DI water, then mounted using DABCO anti-fade reagent. (Due to rapid photobleaching of individual cells, it was not possible to monitor GFP induction in real time using fluorescence microscopy. Therefore fixation and mounting with anti-fade was necessary for confocal imaging). Samples were then imaged on a Zeiss LSM 510-META confocal system mounted on a Zeiss Axiovert 100 inverted microscope. For determination of the time course of GFP expression (Fig. 3), each point represents an average of at least six determinations of approximately one hundred cells each. Fluorescence emission fingerprinting followed by linear unmixing via integrated software was used to separate the fluors from the autofluorescence of the nanostructured silica matrix and confirm the presence of GFP in the cells (Supplementary Fig. 1, C–H). Based on positive and negative

control experiments, it was determined that a 100-fold fluorescence intensity increase over non-stimulated cell background constitutes stimulation. Because samples are fixed at individual time points, we cannot discern unambiguously whether replication takes place but suspect that, due to the confined environment, it does not. This is consistent with endosomal entrapment, as in culture studies of *S. aureus* infected MAC-T cells it was concluded, agr-regulated exoproteins appear to be required prior to the release and replication of *S. aureus* within the infected MAC-T cells⁹.

Viability Determination

To evaluate the viability of individual cells encapsulated in the nanostructured droplets, *S. aureus* strains RN6930 and RN6911 were immobilized as described above. The cells in droplets were then incubated in air at 37°C for the indicated periods of time. At the indicated intervals, samples were removed from the incubator and evaluated using the BacLight (Invitrogen) viability dye set according to the product literature to allow labeling and imaging of immobilized cells. Viability was then determined using a Nikon TE2000 inverted fluorescence microscope equipped with a viability dye filter set from Chroma. Each point represents an average of at least six determinations of approximately one hundred cells each.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Isolation of individual *S. aureus* within a nanostructured droplet. (A) schematic of physical system (not to scale) showing a cell incorporated in an endosome-like lipid vesicle within a surrounding nanostructured lipid/silica droplet deposited on glass substrate and (B) SEM image of physical system. The nanostructure maintains cell viability under dry external conditions and allows complete chemical and physical isolation of one cell from all others. C and D show plan-view optical microscope images of individual cells in droplets (large outer circular areas). Magnified areas show differential interference contrast image and red fluorescence image of individual stained, isolated cells (both C and D) and green fluorescence image of NBD-labeled lipid localization at cell surface (C) or localized pH (D, using Oregon Green pH-sensitive dye). We find that, within the droplet, the cells become enveloped in an endosome-like lipid vesicle (C), and establish a localized pH consistent with physiological early endosomal conditions (~5.5) (D). For further information regarding aerosol assisted droplet formation and lipid localization and pH establishment, see references 16 and 17.

Figure 2.

Auto-induction of quorum sensing and pathogenicity in *S. aureus* strains ALC1743 and ALC1740 isolated within nanostructured droplets. All main images show the merged confocal fluorescence image of a *S. aureus* cell (stained red) within a nanostructured droplet (pseudo-colored blue). Enlarged images show discrete fluorescence channels for the red cell stain, green fluorescent protein production, and a merged image for confirming co-localization of any GFP production by the cell. The absence of GFP production by individual cells at 0hr indicates the RNAPIII pathway has not been activated (A) and the toxin, α -hemolysin, is not being produced (C). However, after ten hours of incubation in air at 37°C, GFP expression can be observed in individual cells showing activation of the RNAPIII-promoted QS pathway (B) as well as expression of secreted virulence factors (D). In (D) we specifically detect activation of the α -hemolysin promoter.

Figure 3.

(A) Percentage of individual cells (or small groups of cells, $n=2-8$, see materials and methods) expressing GFP as function of incubation time at 37° C. (at least 600 cells were counted for each time point). Data are presented as % of cells expressing GFP $\pm$ the 95% confidence interval, with sigmoidal fit only of isolated cell data for clarity. No statistical difference can be seen between individual and small group (2–8 cells) behavior (Chi square analysis). The absence of expression in ALC6513 (an *agrA*(–) mutant containing the same *gfp* reporter, *agr*: P3-*gfp*, as for strains ALC1743, ALC 1740 but lacking *AgrA*, one component of the two component regulatory pair), shows there to be no non-QS induced GFP expression. (B) Percentage of individual cells (or small groups of cells) expressing GFP as function of incubation time at 37 °C for samples prepared with exogenous addition of cyclic AIP1 (AIP) or very low density lipoprotein (VLDL), a proven inhibitor of QS in *S.*

*aureus*²¹. For strain ALC6513, the samples were maintained at 37° C for 24 hours prior to addition of exogenous AIP1 to ensure potential effects of confinement not related to AIP1 concentration would not induce GFP expression. The ALC1743 data without exogenous additions are re-plotted for reference. (C) Viability of *agr*⁺ and *agr*[−] strains of individual *S. aureus* cells isolated within nanostructured lipid/silica droplets: wild type (*agr*⁺) in droplet containing Trypticase Soy Broth (TSB) media (Plot 1), RN6911 (*agr*[−]) in droplet containing TSB media (Plot 2), wild type (*agr*⁺) in droplet without media (Plot 3), and RN6911 (*agr*[−]) in droplet without media (Plot 4). Viability determined using BacLight™ fluorescent viability probe kit. Error bars represent 95% confidence intervals. At least 600 cells were counted for each time point. A statistical difference can be seen when comparing plots of *agr*⁺ and *agr*[−] in droplets with TSB media (P = 0.046, Gehan-Breslow survival analysis).