Confinement-induced quorum sensing of individual *Staphylococcus aureus* bacteria

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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, owing 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 that quorum sensing and genetic reprogramming can 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 that quorum-sensing bacteria 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. The prevailing view is that this signaling allows populations of cells to assess their density—that is, 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 density. 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 location. 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. On this basis QS was proposed 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, investigators 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 QS evolution and maintenance in groups, as ‘cheating’ can occur when individuals exploit secreted group resources without contributing equally to their generation. To reconcile these different perspectives, we hypothesized that QS, independent of its recognized group benefits, can also operate at the single-cell level to provide fitness benefits to individual bacteria that can be selected for. To test this hypothesis, we developed a physical system to isolate individual *S. aureus* and we examined confinement-induced effects on signaling, gene expression and viability. Here we show that self-induction and resultant genetic reprogramming occur efficiently in isolated individual organisms, enabling adaptation and survival. It is noteworthy that, although there are data that suggest that small numbers of intracellular *S. aureus* can undergo QS, and although recent modeling suggests that as few as two cells can induce QS, the ability of a single bacterium to quorum sense in a confined space has not been tested definitively. In fact, to our knowledge no study has ever been performed of any physiologic process in bacteria that could unambiguously examine the behavior of a single bacterium in a confined space.

In *S. aureus*, the accessory gene regulator *agr* operon is responsible for QS regulation. It contains two divergent transcripts (RNAII and RNAIII) driven by activation of two promoters (P2 and P3, respectively). RNAII encodes four genes, *agrB*, *agrD*, *agrC* and *agrA*, that are required to synthesize, export and detect the 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 RNAIII, a regulatory RNA that represses adhesin expression and upregulates an array of toxins, hemolysins, degradatory enzymes and metabolic pathways. Micro-array studies have revealed that 104 genes are upregulated and 34 genes are downregulated as a result of QS, representing ~5% of the genome. Phosphorylated AgrA also induces expression of the RNAII transcript, thus exerting positive feedback control on this regulatory system.

**RESULTS**

**Isolation of *S. aureus* in a nanostructured matrix**

So far, quorum sensing in *S. aureus* has been studied with large numbers of bacteria (1 × 10^6–1 × 10^7) in either broth suspension cultures or cell cultures of phagocyted bacteria. Therefore, the...
potential for individual staphylococci to autoinduce in the absence of neighboring bacteria or cell-signaling inference inherent to these systems (for example, 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) within a matrix fabricated at a sufficiently small physical scale (~20 μm diameter, physically isolated hemispherical droplets; see Fig. 1a,b) so that the overall cell density (~1 cell per 2 × 10^12 μm^3, equivalent to ~0.5 × 10^10 cells ml^−1) exceeded the reported QS threshold (10^5–10^9 cells ml^−1). The matrix was formed by adaptation of our cell-directed assembly approach to an aerosol procedure we developed previously to form ordered cell-incorporated lipid/silica droplet deposited on glass substrate.

**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. (b) Scanning electron microscopy image of the lipid-silica hemisphere confining the cell. The nanostructure maintains cell viabiliy under dry external conditions and allows complete chemical and physical isolation of one cell from all others. (c,d) Plan-view optical microscope images of individual cells in droplets (large outer circular areas). Magnified areas show differential interference contrast (DIC) 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 refs. 15 and 16.

**Monitoring QS of individual, isolated *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 before 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 2** shows representative confocal images of isolated, red-stained ALC1743 immediately following encapsulation and after 10 h of incubation at 37 °C. As seen in the corresponding kinetic plot (Fig. 3a), GFP expression follows a sigmoidal curve. It initiates over 1 hour and increases progressively with time to over 90% at 10 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), thus confirming that our observations were not unique to the laboratory strain RN6390, which 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 threshold. 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 h time course, we observed no measurable GFP expression from strain ALC6513.

**Sensitivity 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)21—was added immediately before the aerosol assembly process. We observed that cyclic AIP1 greatly accelerates GFP expression relative to the corresponding ALC1743 sample prepared without exogenous AIP. In contrast, VLDL suppressed GFP expression for 10 h, after which expression kinetics paralleling those of ALC1743 were recovered. As recently reported, 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, thus 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. **Figure 3b** also plots GFP expression for the agrA− mutant strain isolated for 24 h and then dosed with exogenous AIP1. No GFP expression was observed for a response. Importantly, this architecture—that is, a vesicle-enveloped cell incorporated in a much larger nanostructured silica bead (Fig. 1a,b)—allows individual cells to be maintained in a viable state under externally dry conditions that establish complete physical and chemical isolation of one cell from all others. This reduced physical system is biologically relevant because *S. aureus* is known to become trapped in such intracellular compartments, and it is proposed that they use a QS strategy to induce new gene expression, promoting intracellular survival and/or escape. However, it is presently unknown whether confinement alone can promote QS or whether other factors within the endosomal organelle are required. We used our system to test confinement alone as a mechanism for inducing QS.
and effective localized, integrated concentration of cell-secreted plus exogenous AIP activator or inhibitor responsible for regulation of gene expression.

**Upregulation of virulence factor expression**

Figure 2b,c shows representative confocal images of isolated, individual *S. aureus* strain ALC1740, and Figure 3a shows the corresponding time course of GFP expression. The progressively increasing GFP expression over 10h mirrors that of QS (Fig. 3a) and shows activation of the RNAIII-dependent pathway that induces expression of secreted virulence factors. Here we specifically detected activation of the α-hemolysin promoter. Although there are data that suggest that small numbers of intracellular *S. aureus* quorum sense, the combined data in Figures 2 and 3 provide to our knowledge the first proof of autoinduction of an individual, physically and chemically isolated organism. Additionally, these data provide to our knowledge 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 environments. For both QS and α-hemolysin expression, we observed 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.

**Quorum sensing enhances survival of 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-d 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, Gehan-Breslow survival analysis; compare Fig. 3c, plots 1 and 2). A plausible explanation for the viability difference is that confinement-induced...
QS and attendant upregulation 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 to 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. After 47 h of incubation at 37 °C, cells able to quorum sense had significantly greater viability (Supplementary Fig. 3). It is noteworthy that these experiments, prompted by behavior in our reduced system, are to our knowledge the first to show this agr+ advantage, which is observed for both laboratory strains and recent clinical isolates such as MRSA USA300 (data not shown).

DISCUSSION
By using a reduced physical system devoid of intercellular signaling interference that is inherent to bulk cultures22-24 and previous studies of endosomal entrapment19,20, we have 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 upregulation of the agr effector molecule RNAIII enhances the expression of a diverse array of genes associated with metabolism, transport and virulence25. As implied by our viability studies, one benefit derived by autoinduction is the poising 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 (for example, in the gut or respiratory tract) 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 outset22,23.

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 environment26. 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—that is, cell density, mass-transfer properties and spatial distribution of cells. 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: “that isolated cells should be able to produce enough autoinducer for self-induction under plausible natural conditions”27. But regarding whether autoinducer peptide–controlled genetic reprogramming should be classified as quorum sensing28, diffusion sensing21 or efficiency sensing2, 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 individual29, as we showed in our reduced physical system.

METHODS
Cell lines used. The S. 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 RN6991 (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 stock22,25.

Preparation of nanostructured silica droplets containing live cells. Isolated nanostructured droplets containing individual cells (or small groups of cells; see for example Supplementary Fig. 1) 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 agent13,19,20. Upon evaporation, lipids direct the organization of silica into an ordered lipid/silica membrane, 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 tetrathyloxysilicate (TEOS, from Sigma), ethanol, de-ionized water and HCl (molar ratios 1:4:1.5:10) for 90 min at 60 °C. Water, HCl and TSB (a medium serving as a nutrient required for GFP expression) solution added to the stock to achieve a biologically compatible solution (sol) with a final molar TEOS/ethanol/HCl/water/TSB ratio of 1:4:0:01:6.6. 30 mg ml−1 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.) at a concentration of 100 μM, which exceeds the threshold for induction of QS through exogenous cyclic AIP1 addition13, or VLDL (from US Biological) at 10 μg ml−1. Stocks of the various cell strains (not 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 104 cells ml−1, which is below the quorum-sensing threshold. The solution was immediately aerosol deposited onto glass, resulting in physically and chemically isolated (approximately) hemispherical droplets (Supplementary Fig. 1a) 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. 1b). 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. 1i), similar to that reported previously for our cell-directed assembly process13. 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 (Invitrogen) for 45 min at 37 °C for visualization, washed three times with deionized water, fixed with 4% (w/v) formaldehyde for 45 min, again washed three times with deionized water, then mounted using DABCO (Sigma) antifade 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 antifade 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 100 cells each. Fluorescence emission fingerprinting followed by linear unmixing via integrated software was used to separate the fluoros from the autofluorescence of the nanostructured silica matrix and confirm the presence of GFP in the cells (Supplementary Fig. 1c–h). Based on positive and negative control experiments, it was determined that a 100-fold fluorescence intensity increase over nonstimulated cell background constitutes stimulation. Because samples are fixed at individual time points, we cannot discern unambiguously whether replication takes place, but we suspect that owing to the confined environment it does not. This is consistent with the physical entrapment: in control cultures of S. aureus–infected MAC-T cells, agr-regulated exoproteins appear to be required before the release and replication of S. aureus within the infected MAC-T cells30.

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 100 cells each.

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References


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Author contributions

E.C.G. and D.M.L. conducted all of the experimental work on quorum sensing of isolated individual Staphylococcus aureus. N.P.D. and A.C. constructed the GFP reporter strains. G.S.T. and H.G. conceived of testing quorum sensing of individual bacteria. C.J.B. conceived of the cell-directed assembly and aerosol-assisted self-assembly processes used for nanofabrication.

Additional information

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