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NOTE TO THE EDITOR

Observations of cell cluster hollowing in *Staphylococcus epidermidis* biofilms

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Introduction

Microbial biofilm formation appears to involve complex multicellular behaviours. For example, some bacteria exhibit extensive twitching and swarming motility after association with a surface. These forms of motility appear to be coordinated and to contribute to the spatial organization of biofilm structures (O'Toole and Kolter 1998; Klausen *et al.* 2003). Another intriguing phenomenon is the appearance of hollow interiors in biofilm cell clusters. Such hollowing seems to occur in the later stages of biofilm development. Hollow biofilm structures have been described for *Pseudomonas aeruginosa* (Sauer *et al.* 2002; Webb *et al.* 2003; Hunt *et al.* 2004; Parsek and Fuqua 2004; Stapper *et al.* 2004), *Pseudomonas putida* (Tolker-Nielsen *et al.* 2000), *Pseudoalteromonas tunicate* (Mai-Prochnow *et al.* 2004) and *Actinobacillus actinomycetemcomitans* (Kaplan *et al.* 2003) biofilms. Particularly, striking are movies in which motile cells can be seen seething in the centre of a cell cluster containing many immotile cells (Tolker-Nielsen *et al.* 2000; Hunt *et al.* 2004). Here, we report the direct microscopic observation, by a suite of techniques, of hollow cell clusters in *Staphylococcus epidermidis* biofilms.

Methods

Biofilm formation

Staphylococcus epidermidis ATCC 35984 was grown in glass capillary flow cells as described previously (Rani *et al.* 2005). Biofilms were grown for 48 h at 37°C with continuous flow of one-tenth-strength tryptic soy broth (Difco, Detroit, MI, USA). After two days of development, dense cream-coloured clusters of biofilm were visible in the capillary. There were also large areas of the glass surface that were not visibly colonized. Colony biofilms were prepared as described previously (Zheng and Stewart 2002).

Microscopy

In situ observation of the hydrated biofilm was performed by confocal scanning laser microscopy. The biofilm-containing flow cell was placed on the stage of a Leica TCS-NT confocal scanning laser microscope. Because the capillary tubes were square in cross section, it was possible to interface a microscope objective with the top wall of the capillary and directly observe biofilm through this flat glass wall. The flow cell was injected with 5 mg l^{-1} rhodamine B and allowed to stain for 10 min. The rhodamine B solution was then displaced with a solution of 50 mg l^{-1} sodium fluorescein and stained for 5 min. The specimen was then examined using a 568-nm laser line for excitation of rhodamine B and a 488-nm laser line for excitation of fluorescein. The rhodamine B emission was recorded in a red channel (585–615 nm) and the fluorescein emission was recorded in a green channel (500–550 nm).

Hydrated biofilm structures were also visualized noninvasively by magnetic resonance microscopy (MRM). A capillary flow cell containing *S. epidermidis* biofilm was loaded into the bore of a nuclear magnetic resonance (NMR) magnet and imaged as it grew by applying imaging techniques that have been detailed elsewhere (Seymour *et al.* 2004). Images based on the T2 relaxation parameter, a measure of local water mobility, were prepared.

Some biofilms were stained, embedded and sectioned for examination by epifluorescence microscopy. Biofilms were stained for 2 h with the commercial viability kit *Bac*Light LIVE/DEAD (Molecular Probes, Eugene, OR, USA). These specimens were then cryoembedded by injecting a tissue histopathology medium (Tissue-Tek O.C.T. compound, VWR International, Seattle, WA, USA) into the capillary and freezing it on a block of dry ice. The thin glass was then chipped away from the frozen sample with the aid of a triangular file. The frozen core was embedded in more OCT, then sectioned in a refrigerated microtome. Sections were examined with a Nikon Eclipse E800 microscope using a FITC filter for Syto9 (ex 480/30, DM505, em 535/40) and a G1-B filter (Ex 546/10, DM 575, em LP590) for propidium iodide. Biofilms in capillary flow cells and also colony biofilms were stained for transmission electron microscopy with osmium tetroxide, embedded in a low viscosity epoxy resin, and sectioned as described elsewhere (Zheng and Stewart 2002).

Ultrathin sections were deposited on copper grids and imaged on a Zeiss LEO 912AB electron microscope.

Results

Dense cell clusters, ranging in size from a few tens of microns to several hundred microns in diameter were observed by confocal scanning laser microscopy. In the two colour imaging mode, the biomass appeared red and the bulk fluid appeared green. Rhodamine B sorbs to biomass, staining it red. The fluorescence of fluorescein, in contrast, was quenched in the biomass. Fluorescein thus acts as a negative stain illuminating the bulk fluid. Using this double-staining approach, we discovered occasional cell clusters that appeared to be hollow. These clusters had dark centres in the rhodamine channel (indicating no biomass) while the centre was bright in the fluorescein channel (indicating bulk fluid) (Fig. 1a). Another example of such a hollow cluster has been published in Fig. 5 of Rani *et al.* (2005). Not all cell clusters had hollow interiors. Hollowing seemed to be more common in larger cell clusters compared with smaller clusters.

Hollow cell clusters were also observed by MRM. An example image is shown in Fig. 1c. NMR microscopy does not have the same high spatial resolution that light microscopy does, but we were nevertheless able to discern zones inside the biofilm that appeared to have the same T2 behaviour as the bulk fluid. These hollowed regions were apparent under large cell clusters and they extended to the glass wall. Evidence of hollow cell clusters in *S. epidermidis* capillary flow cell biofilms was also obtained from specimens that were stained, embedded and sectioned. Images from frozen sections examined by epifluoresence microscopy revealed hollow areas, often near the corner of the glass tube (Fig. 1b). Other biofilms were stained for transmission electron microscopy. Cell-free zones in the interior of the biofilm were also evident in these sections (Fig. 1d). The cleared zones viewed by transmission electron microscopy (TEM) were usually located near a corner of the glass capillary.

TEM was also applied to *S. epidermidis* colony biofilms. Colony biofilms are mounds of bacteria cultivated on polycarbonate filter membranes as described by Zheng and Stewart (2002). After 48 h of growth, colony biofilms formed smooth layers of bacteria, approximately 100-μm thick. The cell density and appearance of the cells was quite uniform throughout the depth of the biofilm at this stage of development (see Fig. 3 of Zheng and Stewart 2002). In contrast, after 98 h of growth in colony biofilms, a cleared, cell-free stratum appeared (Fig. 1e,f). This zone, possibly a result of localized cell lysis, was approximately 30 to 40-μm wide and was located in the middle third of the biofilm.

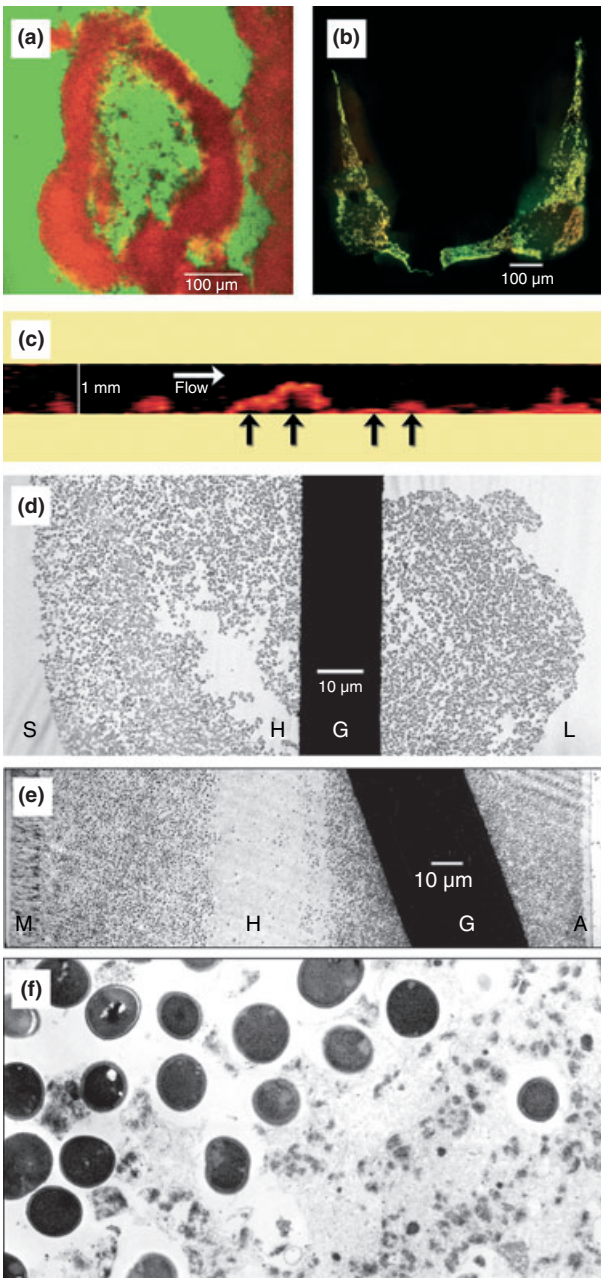

Figure 1 Hollow cell clusters in *Staphylococcus epidermidis* capillary flow cell biofilms observed by confocal scanning laser microscopy (a), *Bac*Light staining and frozen sectioning (b), magnetic resonance micrroscopy (c), and transmission electron microscopy (TEM) (d). Black arrows in panel C indicate putative hollow cluster sites. Layer of lysed cells in a 98-h-old *S. epidermidis* colony biofilm as visualized by TEM (e, f). In panel e, the hollowed stratum in the biofilm is denoted by (H), the membrane supporting the colony biofilm (M), the air interface of the biofilm (A), and the copper grid bar supporting the thin section (G). In panel f, the boundary between the cleared zone and intact cells is shown at higher magnification.

Discussion

These observations collectively support the following model of development in *S. epidermidis* biofilms. In cell clusters that have reached a certain size and age, a lytic process is activated in which regions of the cluster most distant from the bulk fluid experience extensive cell lysis. Lysis creates cell-free zones in the interior of cell clusters. Because *S. epidermidis* is nonmotile, the hollowing observed in biofilms of this bacterium cannot be aided by motility as it seems to be in many of the cases described previously (Tolker-Nielsen *et al.* 2000; Sauer *et al.* 2002; Hunt *et al.* 2004; Parsek and Fuqua 2004; Stapper *et al.* 2004).

Some of the mechanisms of biofilm cell cluster hollowing that have been suggested are activation of a lytic bacteriophage (Webb *et al.* 2003), escape of cells by activation of swimming motility (Tolker-Nielsen *et al.* 2000; Sauer *et al.* 2002; Hunt *et al.* 2004; Parsek and Fuqua 2004; Stapper *et al.* 2004), and release of enzymes that degrade biofilm matrix polymers (Kaplan *et al.* 2003). An autolytic mechanism is plausible in the case of staphylococci, as autolysis is well known in these bacteria (Shockman and Holtje 1994). We examined samples taken from colony biofilms by TEM for the presence of putative viral structures. None was seen.

Possible triggers for the hollowing process in biofilms include quorum sensing (D'Argenio *et al.* 2002; Hunt *et al.* 2003), oxidative stress (Webb *et al.* 2003), nitrosative stress (Barraud *et al.* 2006) and nutrient starvation (Hunt *et al.* 2004). Autolysis in *Staphylococcus aureus* has been shown to be enhanced by high sodium chloride concentrations and to be stimulated by lowered temperature, reduced air supply, and decreased calcium concentration in the medium (Ochiai 1999). Low oxygen concentrations are very likely to prevail in the interior of sufficiently large biofilm clusters.

Is there a function associated with cell cluster hollowing in biofilms? Hollowing might be part of a dispersal process. Although many of the cells in the lysing interior of a biofilm may die, a few others that are liberated from the biofilm matrix may find a path to the bulk fluid. These cells would be carried downstream where they could encounter opportunities to colonize fresh surfaces. The analogy to sporulating, fruiting body-forming bacteria such as *Bacillus subtilis* and *Myxococcus xanthus* is obvious. These bacteria also employ autolysins in the sporulation process. A second possibility it that hollowing is part of a programmed cryptic growth process. The lysis and degradation of cells and extracellular polymers in a cluster interior may serve to release nutrients. These nutrients could then be available to cells in the exterior shell portion of the cell cluster. Perhaps such recycling of nutrients enables new cell activities or differentiation in bacteria that would otherwise remain substrate-limited.

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