

Reorganization of *S. aureus* ECM during Cryo-Preparation for SEM Imaging.

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Biofilms are 3-D communities of bacteria distributed in a highly hydrated extracellular matrix (ECM) [1]. ECM formation and structuring is an important hallmark characteristic of a mature biofilm. Many biofilms, including those of staphylococci, produce ECM that contains polysaccharides, proteins, and extracellular DNA. These substances are all highly hydrophilic, and the water content of a biofilm can be as high as 90%. Here we study 4 methods to prepare hydrated biofilms of *Staphylococcus aureus* (*S. aureus*) for (cryo-)SEM imaging [2]. These different preparation methods give rise to markedly different biofilm morphologies as revealed by cryo-SEM imaging at different stages during ice sublimation. The specimens were all imaged using low-keV electrons in a Zeiss Auriga dual-beam FIB-SEM.

In order of increasing cooling effectiveness, the methods are: (1) drying in air; (2) plunging in liquid nitrogen (LN2); (3) plunging in liquid ethane; and (4) high pressure freezing (HPF) with LN2. LN2 boils at -196 °C (P=1 atm), but plunging a sample into LN2 creates a thin layer of gaseous nitrogen at the sample surface, so heat transfer to the surrounding cryogen is compromised, and the critical cooling rate for water amorphization is typically achieved only for depths from the sample surface of about 1 µm. By surrounding ethane with LN2, it can be cooled below its boiling temperature of -89°C. Gas-layer formation is thus diminished, thereby increasing the cooling rate. Even more effective cooling can be achieved by increasing the pressure. This is the principle underlying the high-pressure freezing technique [3]. Crystalline ice has a lower density than liquid water up to a pressure of 210 MPa. Amorphous ice, however, has about the same density as liquid water. Thus, applying 210 MPa to a sample during freezing counteracts the water expansion during crystallization and reduces the water freezing point to -22 °C. Significantly, at this high pressure, a cooling rate of ~1000 °C/s is sufficient at 210 MPa to vitrify tens of microns of hydrated soft material [4, 5].

Figure 1 shows top-view SEM images of fully dehydrated biofilms after each of the four different preparation methods. Air-dried samples (Fig. 1A) display a collapsed morphology instead of a 3-D gel-like matrix. In contrast, the HPF sample (Fig. 1D) preserves a well-developed biofilm that contains a large fraction of extracellular matrix with no evidence of reorganization due to drying. Between these extremes plunging into LN2 (Fig. 1B) or into liquid ethane (Fig. 1C) preserves the general 3-D biofilm morphology, like that of the high-pressure frozen samples, but does so with significant macro-segregation. The LN2 sample segregates laterally into channels that run perpendicular to the substrate comprising a dense composite of bacteria and ECM separated, after ice sublimation by air/vacuum. The top view of the liquid ethane-quenched specimen (1C) displays only micro/nano porous ECM, but cross-sectional imaging (Fig. 2) shows that this specimen consists of a top layer highly enriched in ECM and a bottom layer concentrated with bacteria. This morphology is most likely a consequence of a liquid/crystal interface that passes from the top surface to the bottom surface during quenching. Significantly, the specimen prepared by high-pressure freezing displays an intimate mixture of bacteria and extracellular matrix material throughout the thickness of the entire biofilm.

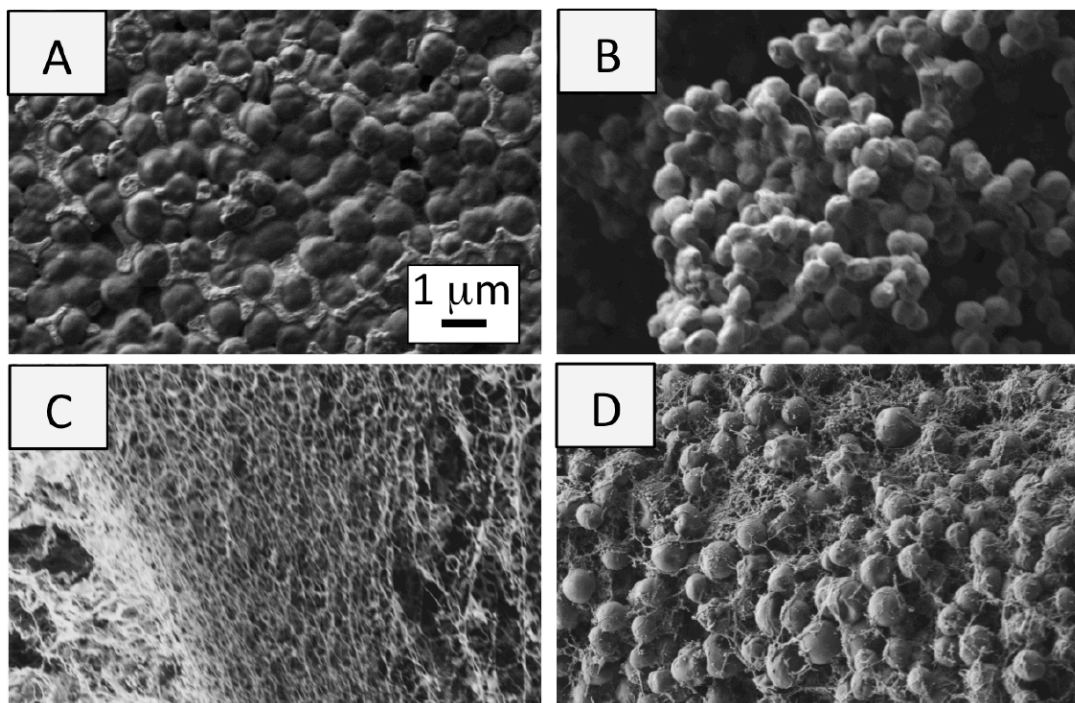


Figure 1. Top-view SEM images of *S. aureus* biofilms after 16 hours sublimation at -105°C . (A) air-dried (no sublimation); (B) plunged in LN₂; (C) plunged in liquid ethane; (D) high-pressure frozen.

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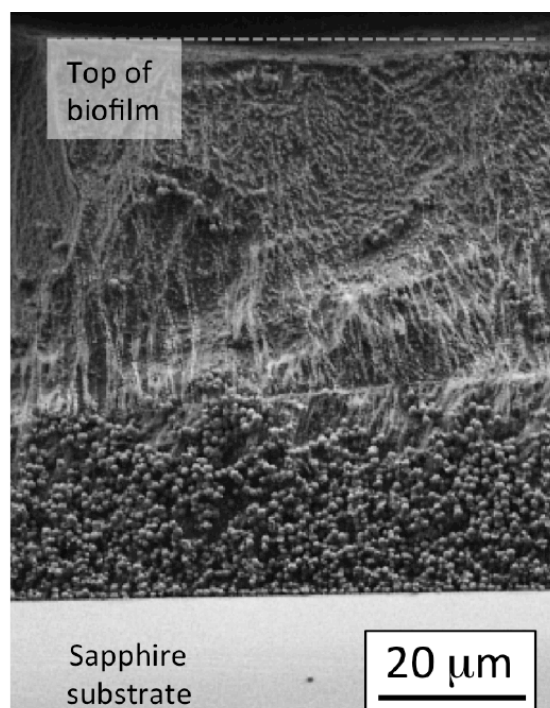


Figure 2. SEM cross-section of *S. aureus* biofilm after liquid-ethane plunge +120 min sublimation shows bacterial macro-segregation.