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The Extracellular Matrix of *Staphylococcus aureus* Biofilms Comprises Cytoplasmic Proteins That Associate with the Cell Surface in Response to Decreasing pH

Lucy Foulston, Alexander K. W. Elsholz, Alicia S. DeFrancesco, Richard Losick
Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, USA

ABSTRACT   Biofilm formation by *Staphylococcus aureus* involves the formation of an extracellular matrix, but the composition of this matrix has been uncertain. Here we report that the matrix is largely composed of cytoplasmic proteins that reversibly associate with the cell surface in a manner that depends on pH. We propose a model for biofilm formation in which cytoplasmic proteins are released from cells in stationary phase. These proteins associate with the cell surface in response to decreasing pH during biofilm formation. Rather than utilizing a dedicated matrix protein, *S. aureus* appears to recycle cytoplasmic proteins that moonlight as components of the extracellular matrix.

IMPORTANCE *Staphylococcus aureus* is a leading cause of multi-antibiotic-resistant nosocomial infections and is often found growing as a biofilm in catheters and chronic wounds. Biofilm formation is an important pathogenicity strategy that enhances resistance to antimicrobials, thereby limiting treatment options and ultimately contributing to increased morbidity and mortality. Cells in a biofilm are held together by an extracellular matrix that consists in whole or in part of protein, but the nature of the proteins in the *S. aureus* matrix is not well understood. Here we postulate that *S. aureus* recycles proteins from the cytoplasm to form the extracellular matrix. This strategy, of cytoplasmic proteins moonlighting as matrix proteins, could allow enhanced flexibility and adaptability for *S. aureus* in forming biofilms under infection conditions and could promote the formation of mixed-species biofilms in chronic wounds.

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Address correspondence to Richard Losick, losick@mcb.harvard.edu. This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Biofilms are surface-associated, multicellular communities in which cells are held together by means of a self-produced, extracellular matrix. The biofilm provides a protective environment that helps shield cells from external stresses and facilitates community behaviors, such as interactions with a host organism and pathogenicity. Both the nature of the matrix and the regulatory mechanisms mediating its production appear to differ widely among bacterial species. Nonetheless, in most cases that have been studied in detail, the matrix consists of a combination of exopolysaccharide, a dedicated protein, and DNA (1, 2).

*Staphylococcus aureus* is an important human pathogen and frequently forms biofilms in clinical settings, most often on catheters and other implanted devices but also in chronic wounds (3, 4). *S. aureus* biofilm formation begins when cells bind to a surface or to host factors, such as fibrinogen and fibronectin (5). The cells then form a multilayered biofilm through intercellular interactions and the production of an extracellular matrix. Initially, it was thought that *S. aureus* biofilm formation relied solely on the production of an extracellular polysaccharide, polysaccharide intercellular adhesion (PIA), the product of genes of the ica operon (6). However, more recent work, including results presented here, indicates that in many strains PIA is dispensable for biofilm formation (7). Extracellular DNA (eDNA), released from cells through regulated autolysis, is thought to contribute to the structural stability of *S. aureus* biofilms (8). Finally, previously determined data indicate that proteins play an important role in biofilm formation, but a clear picture of the identity of the proteins that comprise the matrix has yet to emerge (2, 9).

Here we sought to identify, comprehensively, proteins that comprise the matrix in an unbiased approach based on the use of mass spectrometry. We report that the proteinaceous matrix is principally, if not exclusively, composed of cytoplasmic proteins that are recycled as components of the extracellular matrix during biofilm formation. We further show that the aggregation of these cytoplasmic proteins in the interstitial space around cells takes place in a manner that depends on decreasing pH during growth under biofilm-inducing conditions. We propose a model in which certain abundant cytoplasmic proteins moonlight during biofilm formation as components of the extracellular matrix that mediates cell–cell adherence.

**RESULTS**

Identification of biofilm-associated cell surface proteins. *S. aureus* strain HG003 forms robust biofilms in tryptic soy broth
(TSB) with 0.5% added glucose (here TSBG) (see Fig. S1A in the supplemental material). Both DNA and protein components of the matrix were found to be important for biofilm formation (see Fig. S1A). However, exopolysaccharide and the biofilm-implicated cell wall proteins, protein A (10) and fibrinogen-binding proteins A and B (11), did not contribute significantly (see Fig. S1B).

We took an unbiased approach to identify cell surface-associated proteins present under biofilm-inducing conditions by adapting a 14N/15N metabolic labeling procedure used in a previous proteomic analysis of S. aureus (12, 13). Cell surface-associated proteins were tagged by biotinylation, allowing their specific isolation and identification (13). The ratio of 14N (biofilm) to 15N (nonbiofilm) peptides for each protein gave an estimate of the abundance of a given protein under each growth condition (see Table S1 in the supplemental material).

Interestingly, despite our selecting specifically for extracellular proteins by biotinylation, most of the proteins identified were predicted cytoplasmic proteins. We established a cutoff value for proteins considered to be enriched during biofilm formation as those having a 14N/15N ratio greater than 2. These 11 proteins are characterized by roles in central metabolism, such as glycolysis and fermentation (14). Consistent with our findings, several of these proteins (for example, enolase, transketolase, acetolactate synthase, GAPDH [glyceraldehyde-3-phosphate dehydrogenase], and lactate dehydrogenase) were previously found to be abundant under biofilm-inducing conditions and in the biofilm exopro-"mteome (15–17) in studies that did not investigate their role in biofilm formation. In addition to identifying proteins that were enriched under biofilm-inducing conditions, we also detected extracellular proteins that were either more abundant under the non-biofilm-inducing conditions or showed equal levels of abundance under the two sets of conditions (having a 14N/15N ratio of between 0.5 and 2) (see Table S1 in the supplemental material).

We focused on two of the identified proteins, enolase and GAPDH, and asked whether we could observe association of these proteins with the cell surface by a method that did not depend on biotinylation. Cells harvested from a mature biofilm were washed in phosphate-buffered saline (PBS) at 4°C for 1 h before the cells were removed by mild centrifugation. Among the proteins that were seen in the buffer fraction, by SDS-PAGE (Fig. 1A, lane 1), were enolase and GAPDH, as judged by immunoblot analysis (Fig. 1B).

Do cell surface-associated, cytoplasmic proteins arise from cell lysis during sample preparation? A possible explanation for the presence of cytoplasmic proteins in our cell surface samples was cell lysis or enhanced permeability during enrichment of surface-associated proteins from cells. We took three approaches to investigate this possibility. First, we asked whether cells become permeable upon harvesting from the biofilm and resuspension in PBS using LIVE/DEAD staining. This analysis indicated that the percentage of permeable cells was unaltered for 1 h in PBS (Fig. 2A).

Second, we monitored cell lysis as the decrease in optical density at 600 nm (OD600) with time after resuspension in the presence of Triton X-100, a detergent that stimulates autolytic activity in S. aureus. Resuspension of cells from a mature biofilm in PBS had little to no effect on the rate of lysis, whether stimulated by Triton X-100 or not (Fig. 2B). The rate of stimulated autolysis (decrease in OD600 with time) was very low in cells harvested from the biofilm compared to cells from exponentially growing cultures in the same medium (Fig. 2B), suggesting that biofilm cells are not significantly prone to lysis and that extraction with PBS does not stimulate lysis.

Finally, we asked whether cell surface-associated cytoplasmic proteins arose during biofilm formation or from lysis in PBS. Proteinase K was added upon inoculation, and then a protease inhibitor was added 1 h prior to harvesting the biofilm cells. The results showed that only a very small amount of protein was detected in the PBS extract of biofilm cells (Fig. 2C). When we “spiked in” a cell lysate after the addition of inhibitor, proteins from the lysate were readily detected in the PBS extract, indicating the effectiveness of the inhibitor. The results are consistent with the idea that the cell surface-associated proteins were released from cells during biofilm formation when they accumulated on the outer side of cells and not during our sample preparation procedure. It should, however, be noted that since treatment with proteinase K inhibits biofilm formation (see Fig. S1A in the supplemental material), presumably through degradation of the protein matrix, it is possible that the treated cells could be physiologically different and thus less prone to subsequent cell lysis.

Protein retention at the cell surface reversibly depends on pH. Next, we asked what property of PBS buffer might be responsible for the release of cell surface-associated proteins from cells from the biofilm into the surrounding milieu. We reasoned that a change in pH from the acidic conditions found within biofilms...
to pH 7.5 in PBS might explain our results. In agreement with previous observations, we found that biofilm formation was accompanied by a significant decrease in growth medium pH which was dependent on the availability of glucose (see Fig. S2A in the supplemental material) and that a decrease in pH was required for biofilm formation (see Fig. S2B).

These results led us to hypothesize that cytoplasmic proteins associate with the cell surface to form a protein matrix in a manner that depends on low pH. To investigate this hypothesis further, we incubated biofilm cells in PBS buffer at pH 5. Little to no protein could be detected in the resulting extract, in contrast to the results seen when cells from the same biofilm sample were incubated at pH 7.5 (Fig. 3A, compare lanes 1 and 3). This was not due to protein degradation at pH 5 since proteins could still be extracted at pH 7.5 from biofilm cells that had been incubated at pH 5 for 1 h (Fig. 3A, compare lanes 1 and 2). Proteins remained cell associated at pH 5 but were released upon exposure to higher pH.

We found that proteins added exogenously (not originating from cells within the biofilm itself) could also be “captured” through an interaction with biofilm cells (Fig. 3B). Whole-cell lysate from HG003 cells was added to mature biofilm cell cultures, and the biofilm cells were then incubated at pH 5 and pH 7.5. Proteins (comprising those naturally occurring in the biofilm and those added in) (Fig. 3B, compare lanes 4 and 5) were released from the cell surface at pH 7.5 and not at pH 5 (Fig. 3B, compare lanes 2 and 5). Proteins from exogenously added cell-free lysate were equally recovered from PBS at pH 5 and pH 7.5 (Fig. 3B, lanes 3 and 6), indicating that, in the absence of biofilm cells, proteins do not precipitate at pH 5 but remain in aqueous solution. This suggests that, in the presence of cells from a biofilm, exogenously added lysate proteins interact with the cell surface at low pH and are then released at pH 7.5.

To investigate the specificity of protein interactions with the cell surface, we next determined whether a heterologous protein,
green fluorescent protein (GFP), could interact with the surface of biofilm cells. Cells that had been grown under biofilm-forming conditions were incubated in PBS at pH 5 and pH 7.5 in the presence of lysate proteins isolated from a strain of HG003 that constitutively produces GFP (LCF81). After removal of the cells, GFP was detectable in PBS by immunoblotting with an anti-GFP antibody (Ab) at pH 7.5 but not at pH 5 (Fig. 3C). When cells from PBS at pH 5 were subsequently incubated in PBS at pH 7.5, GFP was released into the higher-pH buffer (Fig. 3C). In contrast, when cells already incubated at pH 7.5 were washed a second time at the same pH, no further GFP could be observed by immunoblot analysis, suggesting that GFP does not associate with cells at pH 7.5 (Fig. 3C). The pH-dependent interaction of GFP with the cell surface was specific to biofilm cells since GFP was detectable in PBS both at pH 5 and at pH 7.5 after incubation with HG003 cells grown to the exponential phase (Fig. 3C). This indicates that a specific feature of cells grown under biofilm-forming conditions is required to mediate the binding of exogenously added proteins to the cell surface.

In the course of conducting these experiments, we observed that cell pellets from biofilm cells formed as diffuse smears (directly from biofilm medium or in PBS at pH 5) at low pH but formed as compact pellets after incubation at pH 7.5 (data not shown). Cell pellets from a biofilm were also more easily resuspended to homogeneity at pH 7.5 than at pH 5. Consequently, we examined cells by phase-contrast microscopy following resuspension. Biofilm cells resuspended at pH 5 were frequently found in large cell clusters that were often associated with other material (Fig. 3D, upper panels). In contrast, cells resuspended at pH 7.5 were largely separate or in small clusters without this associated material (Fig. 3D, lower panels). We propose that low pH causes cytoplasmic proteins to aggregate at the cell surface and that this aggregate is a principal component of the extracellular matrix that holds cells together in the biofilm.

To assess whether the accumulation of cytoplasmic proteins on the cell surface of HG003 biofilm cells is a common feature of S. aureus, we repeated our analysis using four other strains commonly used for the study of biofilm formation. Although they did not make biofilms as robust as those made by HG003, strains Newman, UAMS1, MN8, and RN4220 all produced quantifiable levels of biofilm when grown in 96-well plates in TSBG (see Fig. S3A in the supplemental material); furthermore, the medium pH decreased during growth to around 4.5 to 5 in all cases (data not shown). Cells were harvested from each biofilm and incubated...
in PBS at pH 5 and pH 7.5 for 1 h at 4°C. As seen with HG003, it was possible to observe the release of proteins into PBS from three of the four strains at pH 7.5 but not at pH 5 (see Fig. S3B). Interestingly, RN4220 did not appear to release a significant amount of protein from the cell surface into PBS at pH 7.5 despite making a robust biofilm. This might suggest either that proteins adhere more tightly to RN4220 cells or that this strain utilizes a different mechanism for biofilm formation (7, 18). Taken together, these results indicate that accumulation of cytoplasmic proteins on the cell surface might be a frequent feature of biofilm formation in S. aureus.

**Cytoplasmic proteins associate with the outer side of cells in intact biofilms.** If cytoplasmic proteins accumulate on the cell surface under biofilm-inducing conditions and constitute the extracellular matrix, it should be possible to detect them in intact biofilms that have not been manually disrupted. We used immunofluorescence microscopy to visualize only those proteins accessible to antibodies and thus external to cells. To eliminate nonspecific background caused by the IgG-binding activity of protein A, we utilized a protein A deletion mutant of HG003 (Δspa) which is unaffected in its ability to form biofilms (see Fig. S1B in the supplemental material). As expected, cells of the Δspa mutant did not exhibit binding to a non-specific IgG antibody, anti-Halotag (see Fig. S4A).

Fixed biofilms were probed for target proteins using primary rabbit antibodies and visualized using a secondary antibody conjugated to Alexa Fluor 488. The location of individual cells within the biofilm in a honeycomb-like pattern, again clearly occupying a space distinct from the nuclei/cytoplasm (Fig. 4A). The proteins detected by the two antibodies were most apparent in certain areas and predominantly closer to the top of the biofilm. Fluorescent signal was not apparent using nonspecific antibodies such as an anti-GFP antibody (with cells not producing GFP) or a rabbit anti-mouse-FITC antibody (data not shown).

Since the genes encoding enolase and GAPDH are essential in S. aureus, we were unable to construct deletion mutants that would function as negative controls in immunofluorescence experiments. To confirm that our results were not due to nonspecific binding of anti-enolase and anti-GAPDH antibodies to other cell surface proteins, we constructed Δspa strains in which the native copy of each gene (encoding enolase or GAPDH) was replaced with a copy that would result in a fusion of the HaloTag with the C terminus of the respective protein. The resulting strains (LCF88 and LCF89) were viable and exhibited no significant changes in growth or biofilm formation compared to the Δspa strain (see Fig. S5A in the supplemental material). Furthermore, LCF88 and LCF89 were found to produce bands of the expected size for the fusion products by immunoblotting using an anti-HaloTag antibody in cell lysates and in cell surface extracts (PBS at pH 7.5) of cells grown under biofilm conditions (see Fig. S5B). A commercial anti-HaloTag antibody did not react to proteins on the cell surface of the HG003 Δspa mutant, indicating that the antibody did not display nonspecific binding (Fig. 4B). In contrast, in strains LCF88 and LCF89, respectively, the anti-HaloTag antibody revealed a pattern of protein localization similar to that seen with anti-enolase and anti-GAPDH antibodies (Fig. 4B).

**DISCUSSION**

A hallmark of biofilm formation in certain bacteria is the production of a dedicated protein component of the extracellular matrix, such as Curli in *Escherichia coli* or TasA in *Bacillus subtilis* (19). We
failed to detect an *S. aureus* protein that is unique to the biofilm matrix, although it is possible that such a dedicated protein(s) contributes to the matrix under other conditions or in other strains. Instead, our evidence suggests a model in which cytoplasmic proteins attach to and assemble on the cell surface to form the extracellular matrix. Formation of the matrix is triggered by a decrease in pH during the post-exponential phase in biofilm-inducing medium. We further propose that the matrix thus formed causes cells in the biofilm to adhere to each other. Interestingly, the assembly process is reversible in that proteins can be solubilized from the cell surface, after manual disruption of the biofilm, by artificially increasing the pH. Finally, our evidence indicates that pH-driven association of proteins on the cell surface is a common feature of protein-dependent biofilm formation in diverse strains of *S. aureus*.

How are cytoplasmic proteins released into the surrounding milieu during biofilm growth? It has previously been reported that a variety of species of Gram-positive bacteria, including *S. aureus*, release cytoplasmic proteins into the external environment during stationary phase (20, 21). However, the mechanism by which proteins lacking signal peptides are exported from cells is unclear, and both specific (e.g., secretion) and nonspecific (e.g., cell lysis) mechanisms are possible (20–23). Biofilm formation by *S. aureus* has previously been suggested to be dependent on regulated autolysis for the accumulation of extracellular DNA (24). Conceivably, regulated autolysis may also be the basis for the extracellular presence of cytoplasmic proteins.

The idea that cytoplasmic proteins might accumulate extracellularly and perform a novel function in that location is not new (22, 25). Such “moonlighting” functions of proteins appear to be widespread. In particular, enzymes of glycolysis are commonly performed cytoplasmic proteins in the biofilm matrix might facilitate interspecies interactions. *S. aureus* has been found in chronic wounds (such as diabetic ulcers) with multiple other bacterial species, and 60% of such wounds were observed to contain biofilms (4). *S. aureus* might form multispecies biofilms without the necessity to specifically recognize the dedicated matrix components of the other species. The recycling of cytoplasmic proteins could therefore represent an efficient and versatile strategy for building multicellular communities. It will be interesting to see in future work whether cytoplasmic proteins that moonlight as matrix components represent a widespread strategy for biofilm formation.

**Materials and Methods**

**Strains and growth conditions.** *S. aureus* strains are listed in Table S2 in the supplemental material. *S. aureus* was cultured in tryptic soy broth (TSB; EMD Millipore) and on LB agar (BD) at 37°C. Other growth media were brain heart infusion (BHI; EMD Millipore), BioExpress 1000 (Cambridge Isotope Laboratories), and TSB without glucose (peptone from casein [BD], 17 g/liter; peptone from soymal [Amresco], 3 g/liter; NaCl [Sigma], 5 g/liter; dipotassium hydrogen phosphate [Macron], 2.5 g/liter). *E. coli* DH5α was cultured in LB and on LB agar. Where appropriate, *S. aureus* was selected on 3 μg/ml tetracycline and *E. coli* on 50 μg/ml ampicillin. The pH of biofilm cultures was measured by spotting 20 to 30 µl on pH strips (range, 4.5 to 10; VWR).

**S. aureus** quantitative biofilm assays. For biofilm growth, an overnight culture of HG003 was diluted at 1 in 1,000 into fresh medium (typically TSB supplemented with 0.5% glucose [TSBG] or as specified in the text) and 200 µl was divided into aliquots and introduced into a Nunc MicroWell 96-well microplate (catalog. no. 167008; Thermo/Fisher Scien...
entific). The starting OD_{590} was recorded using a Bio-Tek Synergy II plate reader (Bio-Tek Instruments). Plates were incubated statically at 37°C for 24 h. The medium in each well was removed to a new 96-well plate. The medium in each well was removed to a new 96-well plate. The two washes were amalgamated into a new 96-well microtiter plate. The biofilms were resuspended in 200 µl PBS. The OD_{590} of each fraction was recorded using a Bio-Tek Synergy II plate reader. The starting OD_{590} was subtracted from that for each medium sample, and the absorbance of PBS was subtracted from the values for the wash and biofilm samples. Results from replicate wells (n=4) were averaged, and a standard deviation was calculated. Protease Inhibitor Cocktail (Roche) was used at 80 µg/ml. DNase I (Promega) was added at 10 U/ml. DNase I (Qiagen) was used at 28 U/ml. Proteinase K (Omega Bio-Tek) was used at 0.1 mg/ml. Western blot analysis. Proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 2 h at room temperature in 5% milk powder–5% goat serum (Sigma-Aldrich)–Tris-buffered saline–TWEEN 20 (TBST; 150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween 20). Membranes were probed with primary antibodies diluted in 5% milk–TBST. Rabbit anti-endoamylase antibody (against B. subtilis enolase; kind gift of Jörg Stülke) was used at a dilution of 1:5,000, rabbit anti-GAPDH1 (37) was used at a dilution of 1:10,000, rabbit anti-GFP (38) was used at a dilution of 1:4,000, and rabbit anti-HaloTag polyclonal Ab (pAb) (Promega) was used at a dilution of 1:5,000. Antibody binding was detected with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories) and SuperSignal West Dura chemiluminescent substrate (Thermo/Fisher Scientific).
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