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ORIGINAL ARTICLE

Extracellular matrix structure governs invasion resistance in bacterial biofilms

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Many bacteria are highly adapted for life in communities, or biofilms. A defining feature of biofilms is the production of extracellular matrix that binds cells together. The biofilm matrix provides numerous fitness benefits, including protection from environmental stresses and enhanced nutrient availability. Here we investigate defense against biofilm invasion using the model bacterium *Vibrio cholerae*. We demonstrate that immotile cells, including those identical to the biofilm resident strain, are completely excluded from entry into resident biofilms. Motile cells can colonize and grow on the biofilm exterior, but are readily removed by shear forces. Protection from invasion into the biofilm interior is mediated by the secreted protein RbmA, which binds mother–daughter cell pairs to each other and to polysaccharide components of the matrix. RbmA, and the invasion protection it confers, strongly localize to the cell lineages that produce it.
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Introduction

Bacteria collectively construct spatially complex and functionally diverse communities, termed biofilms, which are now known to be a dominant form of microbial life (Hall-Stoodley *et al.*, 2004; West *et al.*, 2006, 2007a, b; Nadell *et al.*, 2009; Hibbing *et al.*, 2010; Xavier, 2011). Biofilm-dwelling cells secrete extracellular substances, including nutrient-sequestering compounds, digestive enzymes and structural matrices composed of proteins, DNA and polysaccharides (Arvidson, 2000; Visca *et al.*, 2007; Stewart and Franklin, 2008; Flemming and Wingender, 2010; Stewart, 2012). The strain composition and spatial arrangement of bacteria in biofilm communities strongly influence the course of bacterial infections, the functioning of our resident microbiota, bacterial contributions to biogeochemical cycling and industrial bioremediation (Nicolella *et al.*, 2000; Costerton, 2001; Oggioni *et al.*, 2006; Arnosti, 2011; von Rosenvinge *et al.*, 2013). Analysis of bacterial communities in spatial detail poses a challenging problem (Nadell *et al.*, 2013), and thus we are only at the early stages of discovering the ecological and evolutionary principles that underlie the dynamic nature of biofilm composition and development.

Biofilm growth begins when planktonic cells adhere to a surface and initiate secretion of extracellular matrix components (Hall-Stoodley *et al.*, 2004). Growth and division of adherent cells cause biofilms to expand in space, with the resulting multicellular communities acting as sinks for local sources of diffusible nutrients (Stewart and Franklin, 2008; Stewart, 2012). If nutrient sources persist, biofilms can grow into structures many orders of magnitude larger than individual cells. By contrast, if resources become depleted, bacteria can disperse back into the planktonic phase, presumably to encounter new locations with superior resource availability (Kaplan, 2010; Landini *et al.*, 2010; McDougald *et al.*, 2012). The ability of planktonic bacteria to adhere to naked surfaces and initiate biofilm formation has been studied extensively (Beachey, 1981; Palmer *et al.*, 2007). However, the ability of bacteria to invade surfaces already occupied by existing biofilms has received less attention, most notably with respect to intra-specific competition for access to space and resources.

The susceptibility of existing biofilms to invasion by planktonic cells can be expected to influence the spatial and temporal arrangement of cells in biofilms, as well as their strain and species composition (Amarasekare and Nisbet, 2001). These biofilm properties, in turn, will have ramifications on the evolution of bacterial behavior, for example, investment into local competition versus dispersal (Hanski *et al.*, 2011), and potentially for the development of novel probiotic strategies for enhancing or inhibiting biofilms (Preidis and Versalovic, 2009; Rendueles *et al.*, 2013). Invasion dynamics will also influence the evolutionary stability of extracellular matrix production (Xavier and Foster, 2007; van Gestel *et al.*, 2014). The biofilm matrix is

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expensive to produce and confers strong fitness benefits, including tolerance to shear forces, environmental toxins and predation by protists and immune cells (Sutherland, 2001; Flemming and Wingender, 2010; Abee *et al.*, 2011; Kovács *et al.*, 2012; Billings *et al.*, 2013). Secreted digestive enzymes that increase local nutrient availability can also be retained within the biofilm envelope, increasing growth rates in the biofilm microenvironment relative to the surrounding planktonic phase (Sutherland, 2001). The biofilm interior therefore represents valuable niche space into which invading planktonic cells could profit by gaining access.

Here we examine intra-specific biofilm invasion using the model species *Vibrio cholerae*, the etiologic agent of the diarrheal disease cholera. Biofilm production is strongly implicated in *V. cholerae* disease transmission and environmental survival on biotic and abiotic surfaces (Watnick *et al.*, 2001; Kierek and Watnick, 2003; Matz *et al.*, 2005; Nielsen *et al.*, 2006; Fong *et al.*, 2010; Tamayo *et al.*, 2010). We find that biofilms exclude immotile planktonic cells from gaining access to the interior and from remaining bound to the biofilm surface. Motile planktonic cells likewise cannot penetrate to the biofilm interior. Motile cells can adhere to and grow on the exposed surfaces of resident biofilms; however, they are readily removed by fluid flow. Restricting access to the biofilm interior requires the secreted matrix protein RbmA, which binds mother–daughter cell lineages together.

Materials and methods

Strains

Plasmids and *V. cholerae* strains were constructed using conventional cloning and allelic substitution methods (Sambrook *et al.*, 1989; Skorupski and Taylor, 1996). iProof High-Fidelity DNA Polymerase (Bio-rad, Hercules, CA, USA) was used for PCR reactions. Restriction endonucleases, dNTPs and T4 DNA ligase were acquired from New England Biolabs (Ipswich, MA, USA). DNA extraction and purification kits were obtained from IBI Scientific (Peosta, IA, USA) and Zymo Research (Irvine, CA, USA), respectively. A full list of strains and plasmids is provided in Supplementary Table S1. Primer sequences used for plasmid construction are available upon request.

Bacterial strains were derived from *V. cholerae* C6706 El Tor (Thelin and Taylor, 1996). A Δ*hapR* Δ*flaA* double deletion mutant (Nadell and Bassler, 2011), which secretes copious extracellular matrix, was used to produce resident biofilms. We refer to this strain as 'Rugose' (Yildiz *et al.*, 2004), as it forms wrinkled colonies on agar. Invading strain genotypes were: Δ*hapR* (Motility$^+$ Matrix$^+$), Δ*hapR* Δ*flaA* (Motility$^-$ Matrix$^+$), Δ*hapR* Δ*vpsL* (Motility$^+$ Matrix$^-$) and Δ*hapR* Δ*flaA* Δ*vpsL* (Motility$^-$ Matrix$^-$). All strains harbored a chromosomal copy of the fluorescent protein *mTFP1* (Ai *et al.*, 2006), m*KO* (Karasawa *et al.*, 2004) or *mKate* (Shcherbo *et al.*, 2007). In all cases, fluorescent proteins were expressed constitutively.

To delete the matrix protein-encoding locus *rbmA*, we amplified the 1000-bp region immediately upstream and downstream of *rbmA*, joined the fragments by PCR overlap extension, and cloned this 2000 bp fragment into the suicide vector pKAS32 using the KpnI and AvrII restriction sites, producing pCN007. This plasmid was used to introduce an in-frame deletion of *rbmA* to the Rugose strain, yielding R-Δ*rbmA*, which produces resident biofilms lacking RbmA. A Rugose strain harboring an in-frame deletion of the matrix protein-encoding locus *rbmC* was produced in an analogous manner, yielding R-Δ*rbmC*. Complementation of R-Δ*rbmA* was performed by expression of *rbmA* from a plasmid under the control of an arabinose-inducible promoter. This plasmid, pCN013, was made by amplification of the *rbmA* open reading frame and cloning into pYS249 using NotI and EcoRI.

For matrix protein localization experiments, the Rugose strain produced a RbmA 3xFLAG C-terminal fusion (Berk *et al.*, 2012). This strain is referred to as R-RbmA-FLAG. To introduce the FLAG fusion we amplified the 1000 bp sequence preceding the stop codon of *rbmA* and the 1000 bp sequence immediately downstream, using interior primers to introduce a 3xFLAG sequence using PCR overlap extension. This PCR product was ligated into the pKAS32 suicide vector using the AvrI and KpnII restriction sites, producing pCN018. A Rugose strain producing FLAG-tagged RbmC, denoted R-RbmC-FLAG, was produced by an analogous method.

Biofilm adherence and invasion assay

We measured the attachment and ensuing growth of planktonic cells when introduced to environments containing (1) no resident biofilm (naked surface), (2) non-confluent resident biofilms covering ~75% of the substratum, and (3) confluent resident biofilms occupying 100% of the substratum. Experiments were conducted at 30 °C under static conditions using M9 minimal medium containing 0.5% glucose. To generate resident biofilms, overnight cultures of the Rugose strain were back-diluted to an OD_{600} of 1.0, introduced into 96-well glass-bottom microtiter plates (MatTek, Ashland, MA, USA), and incubated for 20 min (non-confluent biofilms) or 1 h (confluent biofilms). Wells were gently washed by three cycles of addition and removal of 150 μl of fresh medium; a final volume of 100 μl of fresh medium was added to the wells, which were then incubated for 24 h. Our inoculation and incubation methods produced three-dimensional resident biofilms whose heights were 20–25 μm at the time invasion assays were performed. For control assays in which microtiter wells contained no resident biofilm, plates were incubated with sterile medium.

Overnight cultures of planktonic cells for use in invasion assays were back-diluted 1:100, incubated at 30 °C for 3 h, and normalized to $OD_{600} = 1.0$. The

liquid phases of microtiter wells (either empty control wells or wells containing resident biofilms) were removed and replaced with 100 µl of cultures containing planktonic cells expressing a different fluorescent protein than that produced by the resident biofilm. Adherence and invasion experiments were replicated with reversed fluorescent protein markers. Planktonic cell cultures were incubated for 1 h with resident biofilms, and the wells were washed as described above. Each biofilm and any adherent cells were imaged by confocal microscopy at 3 µm *z*-intervals from immediately above the well's glass bottom to the maximum biofilm height at 4–9 different locations per well. To assess adherent cells' ability to grow and divide following substratum or biofilm adherence, the plates were incubated for an additional 24 h and imaged following replacement of the liquid phase with fresh medium. For long-term experiments assessing biomass accumulation of invading strains in association with resident biofilms, the procedure above was repeated, but wells were imaged at 0, 24, 48, 96 and 144 h.

Localization of RbmA protein

The R-RbmA-FLAG strain was co-inoculated with the R-Δ*rbmA* strain, or with the Rugose strain. In an accompanying control experiment, the R-RbmC-FLAG strain was co-inoculated with the R-Δ*rbmC* strain, or with the Rugose strain. A Cy-3-conjugated anti-FLAG antibody (Sigma-Aldrich, St Louis, MO, USA) was introduced at 1 µg ml^{-1} for the duration of the biofilm growth period. Biofilms were washed prior to three-color confocal imaging as described above.

Microscopy and analysis

All microscopy was performed on a Nikon Eclipse Ti fluorescence microscope (Melville, NY, USA) fitted with a Yokogawa CSU X-1 spinning disk confocal scanning unit (Biovision Technologies, Exton, PA, USA) and an Andor (Belfast, UK) DU-897 CCD camera. Laser lines at 445, 543 and 594 nm were used to excite the mTFP, mKO and mKate fluorescent proteins, respectively.

Cell counts for all invasion and biofilm competition experiments were obtained using custom MatLab scripts. To quantify resident biofilm and invading cell counts, optical sections from image stacks spanning the entire height of each resident biofilm were taken at 3-µm *z*-intervals to avoid counting the same cells twice. Images were thresholded manually to distinguish fluorescence signal of bacterial origin from background noise. This method, first established by Drescher *et al.* (2014), is particularly critical for discrimination between bacterial cells and noise in the uppermost regions of biofilms. The total bacterial biomass area was summed from these thresholded images and normalized to the experimentally determined average cross-sectional area of single *V. cholerae* cells to calculate cell counts per optical section. Depending on the analysis, cell counts are presented as a function of biofilm height or as totals summed over all biofilm heights, per 100 µm^2 of substratum.

To obtain neighbor distance distributions between cells in Rugose and R-Δ*rbmA* biofilms, representative optical sections of each biofilm type were collected and analyzed manually using native tools in the Nikon NIS-Elements software package. Neighbors were defined as cells between whose centers a straight line could be drawn without passing through another cell. Five hundred such measurements were collected for each biofilm type, and from these values, the average cell diameter was subtracted to obtain distributions of the distances between the outer-perimeters of neighboring cells.

Matrix protein co-localization was performed using the JACoP package for ImageJ (Bolte and Cordelières, 2006). We used Manders' overlap coefficients to describe the fraction of anti-FLAG Cy3 fluorescence that co-localized with RbmA-FLAG producing cells, co-inoculated R-Δ*rbmA* cells, or co-inoculated Rugose cells. An analogous method was used to quantify co-localization of RbmC with producing cells and non-producing cells.

Statistical analysis was performed using Matlab. Because of moderate sample sizes, we used non-parametric methods including Spearman rank correlation and Mann–Whitney *U* tests with Bonferroni corrections for multiple pairwise treatment comparisons.

Results

Resident biofilms resist internal invasion by conspecifics

We assessed whether or not resident biofilms could be invaded as a function of their surface coverage, invading strain motility and invading strain matrix production. A Motility$^-$ Matrix$^+$ strain was used to produce resident biofilms because of its strong extracellular matrix production. The isogenic invading strains were Motility$^-$ Matrix$^-$, or Motility$^-$ Matrix$^+$, or Motility$^+$ Matrix$^-$ or Motility$^+$ Matrix$^+$. Invading strains that were immotile exhibited little attachment or biomass accumulation (irrespective of their capacity for matrix production) in the presence of resident biofilms (Figures 1a and b). This result was especially striking for invading cells that were genetically identical to the resident strain (Motility$^-$ Matrix$^+$; Figure 1b). Specifically, when two cultures of the Motility$^-$ Matrix$^+$ strain differing only in their fluorescent reporter proteins were inoculated together onto open glass, they were competitively neutral, producing biofilms with discrete mother–daughter cell lineage sub-clusters (Supplementary Figure S1) (Hallatschek *et al.*, 2007; Nadell *et al.*, 2010; Nadell and Bassler, 2011). By contrast, when one member of the pair had been present and growing on the surface for 24 h, newly introduced cells of the same strain were completely excluded from biofilm occupation and growth (Figure 1b).

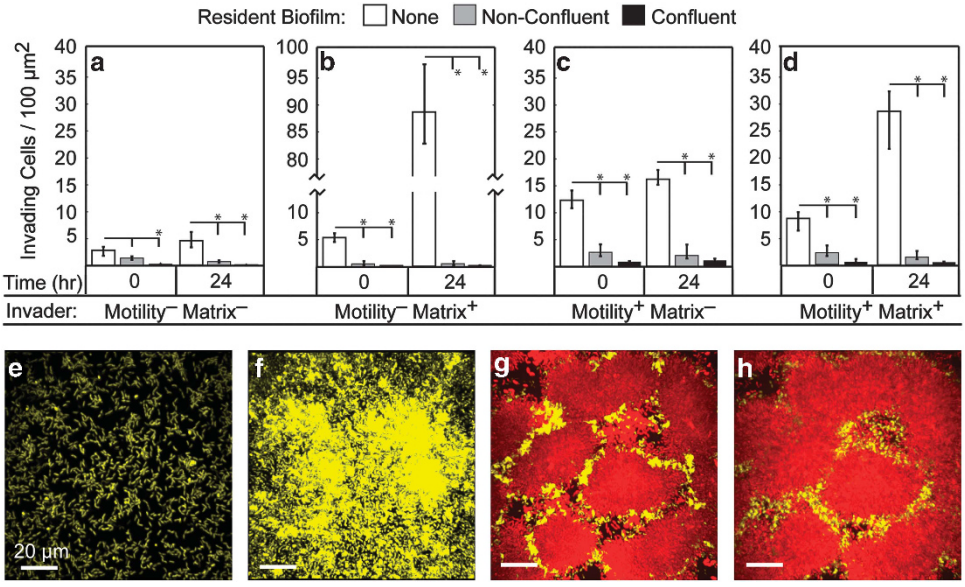

Figure 1 Resident biofilms suppress adherence and subsequent biomass accumulation of planktonic cell populations. (**a–d**) Attachment and growth of invading strains when introduced to open glass substrata or to resident biofilms. Bars denote medians and represent summed cell counts in all biofilm layers per 100 μm² of substratum; error bars denote interquartile ranges with $n = 9$. Asterisks indicate $P < 0.05$ for Mann–Whitney U tests with Bonferroni correction for 16 pairwise comparisons. (**e**) Attachment of Motility$^+$ Matrix$^+$ (yellow) to open glass and (**f**) their biomass accumulation after 24 h. (**g**) Attachment of Motility$^+$ Matrix$^+$ (yellow) to a resident biofilm (red), and (**h**) their biomass accumulation after 24 h. Images in (**e–h**) are top-down views of three-dimensional maximum intensity projections.

Motile invading strains were superior in colonizing biofilms relative to their immotile counterparts (Figures 1c, d), and successful colonization was achieved through flagellar-driven movement, not simply via possession of flagella (Supplementary Figure S2). However, relative to their ability to colonize and grow on unoccupied glass, motile cells were impaired for attachment and biomass accumulation in the presence of resident biofilms (Figures 1c–h). Again, this result did not depend on whether the invading cells could produce matrix. We note that, at initial times, motile invading cells could adhere to the open glass substratum or to the exterior of resident biofilm clusters (Figure 1g). Thus, we suspected that they were removed by mild shear forces imposed while replacing the liquid phase above biofilms prior to imaging. This medium replacement step was performed to distinguish between cells strongly adhered to the resident biofilms and cells loosely associated or in the planktonic phase adjacent to resident biofilms.

To further examine the consequences of medium replacement on invading strains, we performed the invasion experiment as above, allowing the Motility$^+$ Matrix$^+$ strain to colonize a resident biofilm. After 24 h, we imaged biofilms both before and after replacing the wells' liquid phases. Although motile invading cells were able to grow on or near the outer surface of resident biofilms, they were more prone to removal than resident cells by gentle washing because they were over-represented in the wash compared to the resident biofilm strain (Supplementary Figure S3). We conclude that, in the experiments shown in Figures 1c,d and e-h, biomass accumulation of motile invading cells was attenuated because the invading cells were restricted to the biofilm exterior and exposed to removal by shear.

Extracellular matrix structure governs resistance to internal invasion

We wondered how the structural features of the resident biofilm accounted for its resistance to invasion, and if the matrix could be altered to change susceptibility to invasion. Biofilm-dwelling cells of Rugose *V. cholerae* are tightly packed (Berk *et al.*, 2012), a property attributed to the secreted matrix protein RbmA (Moorthy and Watnick, 2005; Fong *et al.*, 2006; Fong and Yildiz, 2007), which is produced rapidly following surface adhesion and becomes distributed throughout mature *V. cholerae* biofilms (Absalon *et al.*, 2011; Berk *et al.*, 2012). Structural studies suggest that RbmA binds mother–daughter cell pairs together, linking them to polysaccharide components of the extracellular matrix (Giglio *et al.*, 2013; Maestre-Reyna *et al.*, 2013). Relative to biofilms expressing the full complement of matrix components, biofilms lacking RbmA exhibit poor sub-cluster coherence (Absalon *et al.*, 2011; Berk *et al.*, 2012). We hypothesized that the absence of RbmA could render *V. cholerae* biofilms susceptible to invasion by planktonic cells.

To determine if RbmA influences resistance to planktonic cell invasion, we introduced motile or immotile matrix producing planktonic cells into microtiter wells containing biofilms produced by

the R-Δ*rbmA* strain (the Rugose strain lacking RbmA). Similar to what we observed for biofilms produced by the Rugose strain possessing RbmA, immotile invading cells displayed little detectable attachment or growth (Supplementary Figure S4). Motile invading cells, on the other hand, exhibited an order of magnitude higher growth in the presence of pre-existing R-Δ*rbmA* biofilms relative to pre-existing Rugose biofilms (Figure 2a). In contrast to Rugose resident biofilms, which restrict invader access to exterior surfaces (Figures 2b and d), resident biofilms composed of the R-Δ*rbmA* strain permitted motile planktonic cells to penetrate throughout the interior (Figures 2c and d). Visual inspection suggested that R-Δ*rbmA* cells were less tightly clustered than Rugose cells within biofilms, possibly permitting passage of planktonic cells to the interior. We confirmed this hypothesis by measuring the distributions of neighbor distances among biofilm-dwelling cells in each strain. Indeed, Rugose cells pack more than twice as closely together as do R-Δ*rbmA* cells (Figure 2e). To confirm the role of RbmA in resistance to invasion, R-Δ*rbmA* biofilms were complemented with inducible *rbmA* on a plasmid (Supplementary Figure S5); introduction of RbmA re-established resistance to planktonic cell invasion into the interior of biofilm clusters.

Invasion protection is privatized by RbmA-producers

Having established that Rugose biofilms are protected from internal invasion by planktonic cells, while R-Δ*rbmA* biofilms are susceptible, we next explored whether the Rugose strain could confer protection to the R-Δ*rbmA* strain in mixed biofilms. We grew mixed resident biofilms by co-inoculating the Rugose and the R-Δ*rbmA* strains at varying initial ratios and challenged their jointly produced biofilms by introducing Motility$^+$ Matrix$^+$ planktonic cells (see Figures 1 and 2). Invading strain growth increased approximately linearly with increasing R-Δ*rbmA* fraction in the resident biofilm (Figure 3a). Microscopy revealed that Rugose and R-Δ*rbmA* cells were spatially segregated into sectors, and that invading planktonic cells integrated into the biofilm interior primarily in sectors occupied by the R-Δ*rbmA* strain (Figure 3b).

Notably, although Rugose and R-Δ*rbmA* cells exhibit similar patterns of growth in liquid culture, Rugose cells are superior in biofilm production and laterally displace R-Δ*rbmA* cells over time when the two are co-inoculated (Supplementary Figure S6). This result was observed both in the presence and in the absence of invading cells, and it is consistent with previous reports documenting that *V. cholerae* biofilms lacking RbmA are structurally weaker than biofilms possessing the full complement of matrix

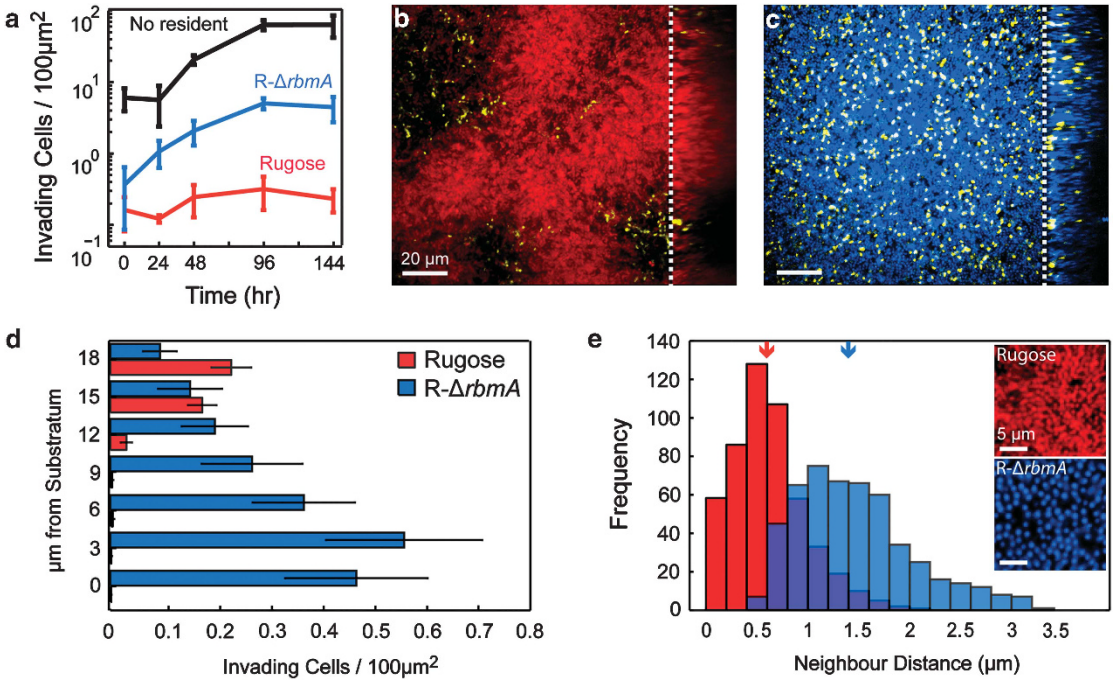

Figure 2 (**a**) Adhesion and subsequent growth of Motility$^+$ Matrix$^+$ cells in the presence of resident Rugose biofilm (red line), resident R-Δ*rbmA* biofilm (blue line), or no biofilm (black line). Data points indicate means and represent summed cell counts in all biofilm layers per 100 μm^2 of substratum; error bars denote the s.d. with $n=4$. (**b**) Rugose biofilm (red) and (**c**) R-Δ*rbmA* biofilm (blue) at 24 h and following challenge by the Motility$^+$ Matrix$^+$ invader (yellow). Central images in (**b**) and (**c**) are single optical planes 6 μm above the glass–biofilm interface, with *z*-projections at the right of each panel. (**d**) Height-stratified profiles of invasion by a Motility$^+$ Matrix$^+$ planktonic population introduced to fully confluent Rugose biofilms (red bars) and R-Δ*rbmA* biofilms (blue bars). Bars denote means and represent cell counts per optical section per 100 μm^2; error bars denote the s.d. with $n=12$. (**e**) Histograms of resident neighbor cell distances in confluent Rugose biofilms (red bars, top inset) and R-Δ*rbmA* biofilms (blue bars, bottom inset), with distribution medians indicated by the arrows at the top of the panel.

proteins (Fong *et al.*, 2006; Absalon *et al.*, 2011; Berk *et al.*, 2012; Giglio *et al.*, 2013; Maestre-Reyna *et al.*, 2013).

Secreted RbmA strictly localizes to mother–daughter cell lineages

The simplest mechanistic explanation for our results is that RbmA is minimally shared between producers and non-producers. We tested this possibility by constructing a Rugose strain producing FLAG-tagged RbmA (R-RbmA-FLAG), and assessed RbmA localization by immunostaining.

Secreted RbmA localized primarily to producing cells when co-inoculated with non-producing R-Δ*rbmA* cells (Figures 4a–e). When the R-RbmA-FLAG strain was grown in the presence of the Rugose strain, which produces un-tagged RbmA, we again observed strong localization of the anti-FLAG antibody to the R-RbmA-FLAG strain (Figures 4a and f–i). This finding indicates that secreted RbmA not only localizes to producing cells, but that it is strongly retained by the mother–daughter cell lineages within which it is being secreted. This result was unexpected in the context of previous studies, which have shown that other protein components of the *V. cholerae* matrix, for example, RbmC, are shared between producing and non-producing cells. To verify that our biofilms behaved in a manner consistent with existing work, we performed the matrix protein localization experiment but paired R-RbmC-FLAG cells with R-Δ*rbmC* cells that produced no RbmC, or with Rugose cells that produced un-tagged RbmC. In agreement with previous studies, and in contrast to how mother–daughter lineages retained RbmA, RbmC-FLAG was shared between producers and non-producers (Supplementary Figure S7). R-Δ*rbmC* cells, again in

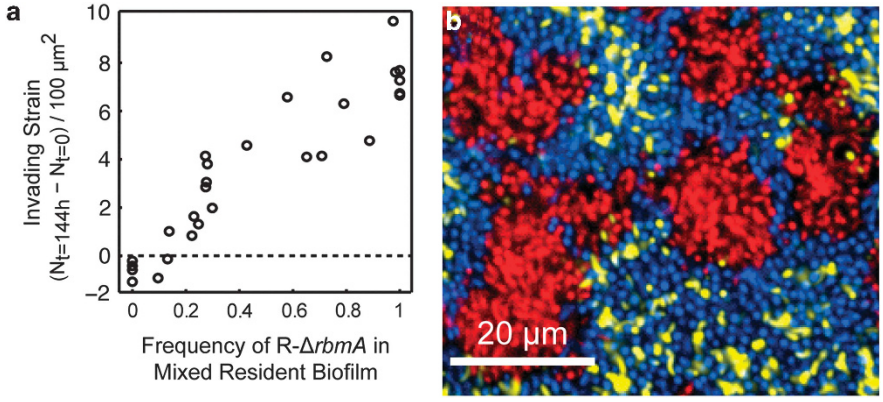

Figure 3 Invasion of resident biofilms comprising a mixture of the Rugose strain and the R-Δ*rbmA* strain. (**a**) Change in invader cell count as a function of the fraction of R-Δ*rbmA* cells within resident biofilms. Data points represent the change in cell count summed over all biofilm layers per 100 μm² of substratum. The horizontal dotted line denotes no change in number of invader cells. Spearman rank correlation: $n = 28$, $\rho = 0.906$, $P < 10^{-7}$. (**b**) A single optical plane 6 μm above the glass–biofilm interface. The resident biofilm consists of Rugose cells (red) and R-Δ*rbmA* cells (blue). Invading Motility$^+$ Matrix$^+$ cells are yellow.

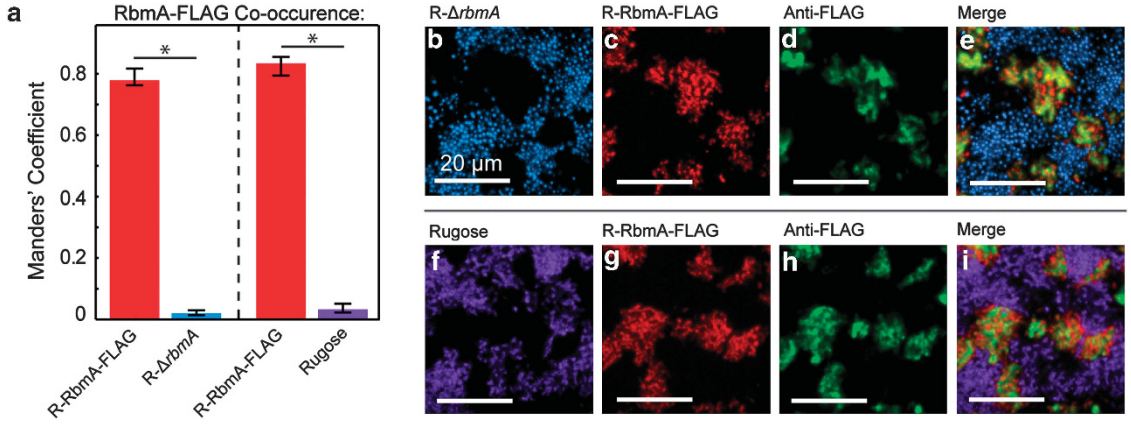

Figure 4 (**a**) Localization of anti-FLAG fluorescence to Rugose cells producing RbmA-FLAG and to co-inoculated R-Δ*rbmA* cells, which do not produce RbmA (left two bars); localization of anti-FLAG to Rugose cells producing RbmA-FLAG and to co-inoculated Rugose cells producing un-tagged RbmA (right two bars). Bars denote medians, and error bars denote interquartile ranges for $n = 6$–8. Asterisks denote $P < 0.05$ for Mann-Whitney U tests. (**b–e**) R-Δ*rbmA* cells (blue), or (**f–i**) Rugose cells producing un-tagged RbmA (purple) were co-inoculated with R-RbmA-FLAG cells producing FLAG-tagged RbmA (red). A Cy-3-conjugated anti-FLAG antibody was used to visualize RbmA-FLAG localization (green).

contrast to R-Δ*rbmA* cells, suffer no competitive disadvantage when co-cultured in biofilms with the Rugose strain (Supplementary Figure S8).

Discussion

Bacterial sociality and biofilm formation play critical roles in microbial ecology, pathogenesis and industrial applications (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Rusconi *et al.*, 2014). The literature often focuses on biofilm formation from the point at which planktonic cells colonize unoccupied surfaces and proceed to initiate biofilm-associated behaviors. However, the lower the frequency of new microhabitat generation and the lower the frequency of environmental disturbances that clear microhabitats of resident microbes, the more often existing habitats will harbor resident biofilms. We therefore aimed to explore the patterns of interaction between *occupied* surfaces and planktonic cells: how does the presence of a resident community change the dynamics of surface colonization and growth by an introduced planktonic population?

We found that mature resident biofilms producing copious extracellular matrix strongly discourage entry of planktonic cells. Immotile invaders, including cells genetically identical to the resident strain, showed almost no detectable colonization or biofilm growth when introduced to previously occupied surfaces. Motile invaders were capable of colonizing the outer surface of resident biofilms, where they could grow but were nonetheless prone to removal by shear. Our findings suggest that biofilms possess an endogenous structure that defends against invading planktonic competitors regardless of their strain or species identity. This ecological view of biofilm behavior is supported by mechanistic studies establishing that *Bacillus*, *Burkholderia*, *Escherichia*, *Pseudomonas*, *Streptococcus* and *Vibrio* species produce polysaccharides during biofilm formation that reduce surface adherence and growth by con- or hetero-specific bacteria (Rendueles *et al.*, 2013; Anderson *et al.*, 2014).

Invasion resistance by resident biofilms, particularly against motile conspecifics, was mediated by exclusion of invaders from the biofilm interior. Previous work suggests that invasion into the interior of a resident biofilm is indeed a competitive strategy employed by planktonic bacteria: several species of *Bacillus* invade both con- and hetero-specific biofilms by tunneling to the interior using flagellar-mediated motility (Houry *et al.*, 2012). The biofilm interior is not always expected to represent favorable niche space, for example, when the bottom layers become oxygen- or nutrient-limited. However, possessing a mechanism that prevents internal biofilm invasion, such as that described here for *V. cholerae*, could be important during competition between biofilm-dwelling and planktonic cells in natural scenarios when the biofilm interior is advantageous for growth or survival.

We suggest that invasion resistance will be especially strongly selected whenever fitness-enhancing solute concentrations are high within the biofilm interior, such as may occur when nutrients are derived from the substratum. An example of this scenario was recently described for *V. cholerae* growing on the biopolymer chitin. Wild-type *V. cholerae* cells, which secrete chitinases that liberate soluble nutrients from chitin substrata, are vulnerable to exploitation by chitinase non-producers (Drescher *et al.*, 2014). Rugose *V. cholerae* strains, which generate thicker biofilms than their wild-type counterparts, avert exploitation and render chitinase production evolutionarily stable. The additional cell layers present in biofilms formed by Rugose *V. cholerae* strains consume essentially all nutrients liberated from the chitin substratum before they can diffuse into the surrounding environment, greatly reducing nutrient availability to exploitative non-producers. This mechanism promoting the evolutionary stability of chitinase production depends on exclusion of foreign cells from the interior of chitinase-producing biofilms, where the nutrients produced by chitinase activity are at their highest concentrations.

Here we have documented that foreign cells are indeed excluded from biofilms by Rugose *V. cholerae*; invasion resistance hinged upon production of the matrix component RbmA, which binds mother–daughter cells together and links them to polysaccharide components of the extracellular matrix. RbmA-mediated invasion protection was not shared between RbmA producers and non-producers, and the RbmA protein localized tightly to the mother–daughter cell lineages that secreted it. This result was somewhat surprising, as RbmA-deficient biofilms can be complemented by exogenous addition of purified RbmA (Absalon *et al.*, 2011). Furthermore, other protein components of the *V. cholerae* matrix, including RbmC and Bap1, are shared between producers and non-producers (Absalon *et al.*, 2011; Berk *et al.*, 2012). Together with previous reports, our results support a model in which RbmA is strongly retained through interaction with the cell exterior and with extracellular polysaccharides, which are known not to be shared among producers and non-producers. In this model, secreted RbmA is tightly retained by the cell lineages that produce it, which privatizes its use, while exogenously supplied RbmA can integrate into the matrix surrounding non-producing cells and thereby complement their deficiency (Absalon *et al.,* 2011).

The mechanism we describe underpinning *V. cholerae* invasion resistance supports a perspective in which cell lineages capture substratum area and defend their 'territory' within growing biofilms, selectively benefiting clonemates in the process (Mitri and Foster, 2013). This ecological interpretation is consistent with recent studies

suggesting that bacterial phenotypes as fundamental as cell shape contribute to optimizing surface occupation (Persat *et al.*, 2014), that competing strains attempt to displace one another from occupied substrata (Nadell and Bassler, 2011; Houry *et al.*, 2012; Schluter *et al.*, 2015), and that cell lineages vie for access to areas harboring the highest nutrient concentrations within biofilms (Xavier and Foster, 2007; Nadell *et al.*, 2008; Kim *et al.*, 2014). A recent report further supports the generality and ecological realism of our results. After introducing two differently colored but otherwise identical strains of wild type *V. cholerae* into the intestinal tracts of infant mice, Millet *et al.* (2014) observed biofilms made of clusters that remained mono-colored for the duration of the infection. This finding provides *in situ* support for our primary result, namely that *V. cholerae* biofilms are composed of tightly coherent clonal lineages that discourage the entry of foreign cells.

It is important to note that there are circumstances in which planktonic cells can adhere to and grow within resident bacterial communities. This phenomenon has been particularly well studied in the context of oral microbial biofilms (Kolenbrander *et al.*, 2010). Previous work has identified patterns of inter- and intra-generic cell aggregation that determine a characteristic order of addition of species to microbial communities on teeth after cleaning. The observation of co-aggregation partnerships may indicate instances of mutualism in which both members of the pair benefit from close spatial association (Mitri *et al.*, 2011). However, it is also possible that different bacterial species are simply adapted to bind and subsequently outcompete resident biofilms in communities that have a consistent sequence of occupant eco-types, analogous to competition between late-successional and early-successional species in patches of disturbed forest (Guariguata and Ostertag, 2001). An important ongoing effort in biofilm research is to identify the relative contributions and spatiotemporal scales of cooperative and competitive interactions that govern biofilm composition, structure and function.

Conflict of Interest

The authors declare no conflict of interest.

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