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Uncovering the Role of Biofilm Matrix Proteins in *Vibrio Cholerae* Biofilm Formation

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by

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ABSTRACT

Vibrio cholerae biofilm formation is central to both pathogenicity and environmental persistence. The matrix proteins RbmA, Bap1, and RbmC along with Vibrio-exopolysaccharide (VPS) provide the foundation for biofilm architecture. Through genetic analysis and extracellular complementation assays, we demonstrate that Bap1 and RbmC both contain VCBS and Lectin domains that discretely contribute to biofilm formation. We determined that Bap1 and RbmC both interact with RbmA, providing an explanation for their functional redundancy. Further characterization of interactions of matrix components revealed that Bap1 but not RbmC can interact with VPS. We also analyzed posttranslational regulatory controls on matrix protein stability and found that HapA is the primary protease responsible for degradation of Bap1 and RbmC. To understand how matrix interactions impact protein stability, we identified that biofilm architectural components contribute to protease activity, indicating that structural integrity potentiates matrix proteolysis. These findings fill a crucial knowledge gap regarding biofilm formation and the subsequent degradation during *Vibrio cholerae* biofilm formation.

This thesis and the work within are dedicated to my late father Thomas Kiblen and my mother Cynthia Micinski for their support and compassion. These pages would be left unwritten without her exhaustive dedication as a single mother since 2008.

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Chapter 1: Introduction

Joseph Kiblen and Fitnat H. Yildiz

Overview

Bacteria exist primarily in one of two forms, free forming planktonic cells, or in a sessile biofilm state sheathed within a matrix. Biofilms are matrix encased microbial aggregates that are able to adhere to biotic and abiotic surfaces. Biofilm matrices are typically entrenched by excreted molecules termed extracellular polymeric substances (EPS) that is characteristically composed of exopolysaccharides, extracellular DNA, proteins, and lipids.[1] Biofilms themselves provide a unique medium that facilitates horizontal gene transfer, pathogenicity, and intercellular communication delivering a fitness advantage to species in competitive microbial environments.[2] Biofilms also provide a physical advantage as the matrix sequesters the lysed cellular components to provide a reservoir for other cells to use, as well as a physical barrier to limit the effectiveness of antimicrobial agents including antibiotics, bacteriocins, and bacteriophage. EPS comprise the majority of organic carbon within biofilms, account for up to 90% within the matrix.[3] Bacterial polysaccharides are extracellularly secreted sugar repeats that can be heterogenous or homogenous in composition.[4] Exopolysaccharides use various α and β glyosidic linkages to control rigidity, hydrophobicity, and solubility which affect each exopolysaccharide's role within the biofilm.[5] Extracellular DNA or eDNA is another EPS substance which has been linked to chelation of cations such as magnesium, cationic exopolysaccharides such as PSL, and adhesion to abiotic surfaces.[6] Lipids within the biofilm matrix are found as free lipids, glycolipids, and biosurfactants which have been shown to assist with surface tension, temperature adaptation, and partly in biofilm dispersion, although the study of matrix lipids is limited.[7–9] Biofilm

matrix proteins are one of the most well characterized EPS with roles in biofilm matrix assembly, nutrient acquisition, and enzymatic functions.[10–12] Biofilm proteins which impact biofilm architecture may exert their impact at different stages of development.

The bacterial biofilm lifecycle consists of three main stages: (i) initial attachment to a biotic or abiotic surface, (ii) accumulation of the cells composing the biofilm including cell proliferation, cell-cell adhesion, and development of a mature biofilm, and (iii) detachment/dispersal of the biofilm.[13] Each of these stages utilizes a different subset of biomolecules to carry out a specific function from regulation of exopolysaccharide synthesis to mediating detachment from adhesion proteins, thus the biofilm lifecycle presents a unique feature of microbial life to better understand how bacteria have thrived for over 3.5 billion years.

Vibrio cholerae

The facultative anaerobe *Vibrio cholerae* is responsible for the diarrheal disease cholera which causes estimated 2.9 million infections and 95,000 deaths annually.[14] Biofilms are an essential component of the *V. cholerae* lifecycle as a requisite for environmental and host persistence, transmission, and dissemination.[15] The bacterial disease cholera are described as early as 5th century BC, however, the organism was first isolated in 1854.[16] There are seven recorded cholera pandemics, where the 7th is ongoing predominantly in Asia and Africa.[17]The current outbreak is caused by the O1 El Tor biotype where an increase in biofilm formation capacity has been observed.[18] The infection cycle begins with ingestion of the organism often

through contaminated food or water. Once infected, the diarrheal disease may cause dehydration, and in some cases mortality.

Adhesion

Adhesion has become a central target for combating biofilm mediated and nosocomial infections, as if bacteria are unable to adhere to surfaces, they are unable to institute an infection. Since the establishment of the biphasic lifecycle of bacteria in planktonic and biofilm-associated states, elucidating the mechanisms by which bacteria undergo this phasic transition is crucial to understanding ways of combating infection.

The initial stage of bacterial attachment can be mediated through abiotic and biotic surfaces, where multiple proteins may serve similar functions. *V. cholerae* utilizes multiple proteins for surface adhesion in abiotic and biotic environments were multiple proteins may fulfill similar functions. Surface attachment is a complicated process that is often mediated by multiple extracellular polymeric substances, however, pili, flagella, and exopolysaccharides are three which have been implicated in attachment. Partial functional overlap has been observed between the mannose-sensitive hemagglutinin (MSHA) type IV pili and the N-acetyl glucosamine (GlcNAc)-binding protein A (GbpA) where both are involved in aquatic surface attachment.[19] Though GbpA and MSHA participate in similar attachment processes, their effects differ, as GbpA mediates intestinal colonization, as well as adhesion to epithelial cells, chitinous and environmental surfaces.[20,21] In contrast, deletion of MSHA type IV pili does not impact colonization.[20,21] MSHA type IV pili is involved

in flagellar coordination to alternate between roaming to orbiting motility modalities important for irreversible attachment and microcolony formation.[22]

Biofilm Matrix Maturation in *Vibrio cholerae*

The *V. cholerae* biofilm matrix contains three recently discovered proteins, RbmA, Bap1, and RbmC as well as the *Vibrio*-exopolysaccharide VPS.[23] The production of VPS requires most of the 18 genes in the *vps*-I and *vps*-II clusters, with the exception of *vpsC* and *vpsQ*.[24] Additionally, VPS production along with the matrix protein RbmA provide an *in vivo* infection advantage. RbmA is a biofilm scaffolding protein that is important for the development of biofilm architecture.[25,26] RbmA binds to VPS through its fibronectin-III domain in which RbmA initially acts as a dimer.[27] Once RbmA binds VPS it then forms a higher order structure. Binding to VPS is crucial to biofilm formation as RbmA serves as a tether that forms flexible linkers between the cells and the extracellular matrix.[28]

Biofilm-associated protein 1 (Bap1) is a shared communal matrix component important for cell-surface adhesion, antimicrobial resistance, osmotic expansion, along with pellicle maintenance and hydrophobicity.[29–31] Bap1 contains two VCBS domains, a putative calcium binding site, along with one Lectin domain with uncharacterized targets. Bap1 is secreted by a founder cell and then localizes at the cell-surface interface as well as encasing cell clusters with the third biofilm matrix protein, RbmC.

Rugosity and biofilm structure modulator C (RbmC) contains two VCBS domains, a putative calcium binding site, along with two Lectin domains which have

been implicated in binding to extracellular eukaryotic glycans.[32] In mature biofilms, RbmC is localized at cell clusters along with Bap1 and has been shown as a requisite for sustained VPS incorporation into the matrix.[11] VCBS domains are believed to bind to an unidentified ligand during biofilm matrix formation.

Bap1 (VC1888) and RbmC (VC0930) exhibit with 47% sequence homology and they are localized at similar areas in the biofilm matrix where their roles in biofilm formation may be partially redundant.[23] The deletion of either *bap1* or *rbmC* reduces biofilm formation with a greater reduction observed by deleting *bap1*. Surface attachment has been investigated with Bap1and RbmC where primarily Bap1 and to some extent RbmC contribute to abiotic surface adhesion.[31,33] However, under dynamic flow conditions, the single deletion of *bap1* or *rbmC* results in little defect of mature biofilm formation, whereas the deletion of *bap1* and *rbmC* is unable to maintain adherence indicating that matrix proteins are required for biofilm formation, however, their specific role is unknown.[23]

VCBS Domains and Biofilm Formation

Though matrix proteins Bap1 and RbmC are thought to have similar functions in the matrix, there is some deviation in their role during biofilm formation. It has been shown that RbmC has an important role for VPS integration into the matrix, as well as VPS being necessary for retention of biofilm matrix proteins RbmA, Bap1, and RbmC.[11] Although *V. cholerae* requires RbmC for sustained incorporation of VPS into biofilms, the mechanism of action and binding capacity to the polysaccharide is unknown. It has been suggested that RbmC's Lectin domains do not

bind VPS due to their perceived inability to accommodate the α1,3- α1,6- linked branched carbohydrate moieties due to glycan array analysis.[32] Because VPS consists of α- and β1,4-linkages of glucose and galactose monomers, it is believed that the steric hindrance between the interacting residues and the exopolysaccharide would prohibit binding, although direct interactions with VPS have yet to be tested for Bap1 and RbmC.[34]

There are other VCBS containing proteins, in which many have been predicted to be involved in adhesion due to similarity to previously characterized biofilm surface proteins.[35] These proteins are similar to orthologs of Bap1 and RbmC of *V. cholerae* due to their conservation of acidic residues and threonine-rich regions (http://eggnogdb.embl.de). The conservation of these residues has been identified in integrin conserved sequences including FG-GAP domains which have been identified bioinformatically in Bap1 and RbmC.[36,37] Integrin signatures have been implicated in fibronectin interaction with integrin domains, such as with the conserved RGD and LDV motif in fibronectin domains, though the consensus residues in Bap1 and RbmC remains unclear.[38–40]

It has shown that bacterial integrin domains are highly conserved among one another, especially in the presence of predicted cation binding motif.[37] It is believed that domain prediction services may annotate FG-GAP containing proteins as integrin due to the number of different consensus sequences. These findings may suggest that integrin-type interactions could potentially explain established protein-protein interactions such as the LDV dependent interaction of OmpT with Bap1.[41]

VCBS domains are found within biofilm matrix proteins Bap1and RbmC, though the domains has become increasingly common outside of *Vibrio, Colwellia, Bradyrhizobium, and Shewanella* species. One such example is BrtA, a biofilm-associated adhesin expressed during host infection by *Bordetella bronchiseptica* which contains pairs of the VCBS dystroglycan-type cadherin-like unit, the von Willebrand Factor A domain (vWFA), RTX motif, and type I secretion target signal.[42] The *B. bronchiseptic* RB50 and S798 strains contain BrtA proteins with up to eight VCBS-dystroglycan-type cad-like unit repeats.[43,44] VCBS-dystroglycan-type cad-like unit repeats are also found in *B. parapertussis* and in *B. pertussis*.[44] Different forms of BrtA within *Bordetella* vary in size, with some differing in their number of VCBS repeats. Deletion of VCBS repeats in BrtA impaired biofilm formation with partial retention of bacterial-substrate adherence, however, deleting both VCBS and von Willebrand Factor A domains resulted in no surface adherence or biofilm formation implicating the role of VCBS domains within biofilm formation. BrtA surface adhesion and subsequently BrtA-mediated biofilm formation was found to be Ca^{2+} dependent. Other VCBS containing proteins including Bap1 and RbmC also contain Ca^{2+} binding sites, however, it is unclear whether VCBS domains directly involve with Ca^{2+}.[45]

Other proteins involved in biofilm formation include the VCBS domains repeat protein in *S. oneidensis* which shows resemblance to the *P. fluorescens* adhesin LapA.[31] The *Anabaena* sp. PCC 7120 biofilm matrix protein HesF contains VCBS domains that contribute to surface adhesion, cell-cell aggregation, and exopolysaccharide retention.[46]

There are similarities between VCBS containing proteins BrtA, HesF, and *S. oneidensis* VCBS-protein as cell-surface and cell-cell adhesion proteins. The VCBS annotation under PFAM (PF13517) and TIGR (TIGR01965) database includes a carbohydrate-binding Lectin bound to N-acetylglucosamine, however, their involvement in biofilm formation remains elusive. Thus, VCBS domains are found within a diverse population of proteins important for biofilm formation, however, the mechanism explaining their impacts on biofilm formation remains unknown.

Biofilm Matrix Development in Other Species

Biofilm matrix proteins that help to form a mature biofilm architecture are found in a plethora of other organisms such as *P. aeruginosa*, *B. subtilis*, and *S. aureus*. Many biofilm matrix proteins have homologs to one another which highlight how many organisms have adopted similar measures to conduct biofilm formation. For example, *P. fluorescens* biofilms utilize LapA, a cell localized adhesion protein while *P. aeruginosa* utilizes the adhesion CdrA. LapA is released by the periplasmic cysteine protease LapG in low phosphate conditions, under high phosphate conditions LapA is un-cleaved and maintains surface attachment, and a deletion of LapG results in a 2-fold increase in LapA and increased biofilm thickness.[47,48] *P. aeruginosa* contains CdrA, an adhesin known to bind PSL, one of the exopolysaccharides produced by *P. aeruginosa*. [49] Interestingly, the protease LapG has a homolog in *P. aeruginosa* that targets CdrA for biofilm dispersal, however, this release requires the presence of PSL.[50] Similarly, in *B. bronchiseptica* its LapA protein BrtA is released

by its LapG homolog which is 48% identical to *P. fluorescens* LapG that is regulated by its LapD homolog which is 30% identical to *P. fluorescens* LapD.

P. fluorescens adhesion protein LapA promotes biofilm formation in its un-cleaved, while cleaved LapA does not. However, cell-associated and secreted forms of CdrA promote biofilm formation and interact with PSL, a major distinction between CdrA and LapA function.[51] Matrix protein interaction with PSL much like VPS produced by *V. cholerae* is hypothesized to be a noncommunal resource which can still provide benefit to non-EPS producing cells in the form of biofilm protection.[52,53]

Bacillus subtilis is a Gram-positive soil spore forming bacterium known for its beneficial relationship in protecting plants in a biofilm dependent manner.[54] *B. subtilis* contains two major proteins within the matrix, the major protein being TasA which forms fibers that stabilize the biofilm matrix, and BslA, a secreted protein that forms the hydrophobic coat around the biofilm which renders it water-repellent. The bacterium forms biofilms in a which monomeric BslA (previously YweA) is both necessary and sufficient for formation, although the dimeric form is implicated in the hydrophobic state of the biofilm.[55] The oligomeric state of the protein is dependent on the redox environment within the soil such that in an oxidizing environment a dimer is formed, whereas in a reducing environment a monomer is observed. Oligomerization is enabled by the requisite of two cysteines, C178 and C180 in which disulfide bond formation is mediated the oxidoreductases BdbA and BdbD. BslA's structure has been solved in which the hydrophobic cap forms immunoglobulin-like

fold, however, BslA is not the only major secreted protein as TasA and TapA also play sufficient roles in the biofilm matrix.[56]

TasA produces amyloid-like fibers that give structure to the matrix encoded by the *tapA-sipW-tasA* operon where SipW cleaves the signal peptide from TasA and TapA.[57] TapA although produced at a 1:100 ratio relative to TasA is required for TasA fiber anchorage to cells and for appropriate fiber assembly.[58] Though often oligomerized, recombinant TasA with and without its signal peptide were found primarily as monomers, indicating that oligomerization may be mediated by an unknown signal. The polyproline II helices may be responsible for fibril formation, as the motif is involved with fibrillar diseases, and protein elasticity.[59,60] Monomeric TasA forms a globular structure presenting a fundamental difference between other well studied functional microbial amyloids such as CsgA from *E. coli* and FapC from *Pseudomonas*.[61,62] It is unclear whether fibrilization of TasA occurs with secreted and folded TasA or whether the protein directly fibrilizes once secreted in an unfolded form. Fibrilization of TasA may be nucleated by TapA, in a manner similar to CsgA and CsgB in *E. coli* as amino acids 50-57 of TapA have been shown to be required for proper fibrilization.[57] Amyloid-like protein fibrilization may also use other factors for nucleation, as in *C. albicans* force-induced nanodomain propagation produces self-propagating amyloids of Als5p.[63] Though TapA's five cystine residues contribute to wild-type biofilm architecture (corrugation pattern), they are dispensable for fibril polymerization. Biofilm surface adhesion requires the presence of the *tapA-sipW-tasA* operon, however, adhesion was unaffected by the loss of only *tasA*.[64] This is in contrast to adhesion in multi-species biofilms with *S.* mutans, where deletion of *tasA*

significantly reduced adhesion force, indicating that TasA and its fibers may facilitate adhesion in multispecies biofilms.[65]

Dispersal

The final stage of film development is termed dispersal in which the mature biofilm is disbanded providing an opportunity for cells to colonize other local or distant locations. Biofilm dispersal has long been studied in terms of environmental and biological signals including antimicrobial compounds, quorum-sensing signals, nutrient availability, and matrix-degrading enzymes, however, no mechanism is ubiquitous.[65]

Exopolysaccharide Matrix Dispersion

One common mechanism to facilitate dispersal is to dismember the polysaccharide mesh encasing the bacteria through utilization of enzymatic degradation. One of the most well characterized biofilm dispersal proteins dispersin B is a glycoside hydrolase which cleaves the polysaccharide PGA.[66] Dispersin B or DspB is found within *A. actinomycetemcomitans* and has the ability to dissolve its biofilms as well as those of *Y. pestis, E. coli*, *P. fluorescens*, and *S. epidermidis* through hydrolysis of the common poly-β-1,6-GlcNAc glyosidic linkage.[67] DspB cannot cleave β-1,4 bonds although other hexosaminidase such as *S. plicatus's* 1HP5 perform this function due to the absence of Trp408 that is found in BspB presenting an explanation for the difference in substrate toleration[66,68]. In *P. aeruginosa* PEL and PSL are vital exopolysaccharides that require processing for their incorporation into

the matrix by the PelA and PslG glyosidic hydrolase encoded within their respective operon.[69] Exogenous $PelA_h$ and $PslG_h$, the hydrolytic domains of the respective enzymes, contained the ability to inhibit biofilm formation and disrupt established biofilms potentiating antibiotics and enhancing bacterial elimination by mammalian immune system.[70] Dispersal mediated by glyosidic bond cleavage presents an attractive approach for thwarting biofilm persistence.

Extracellular DNA Dispersion

Extracellular DNA has been shown to play a role biofilm architecture in a number of pathogenic bacteria such as *P. aeruginosa, H. influenzae*, and *S. aureus*.[71–73] Extracellular DNA or eDNA functions as an intercellular connector to stabilize biofilm formation, thus providing a target for facilitating dispersal. Nontypeable *Haemophilus influenzae* (NTHI) contains double stranded DNA within its matrix as well as a recently discovered calcium dependent nuclease termed Nuc which is homologous to *Staphylococcus aureus* thermonuclease.[74] Nuc activity facilitates even bacterial distribution throughout the biofilm as *nuc* deletion resulted in increased eDNA, large cellular aggregation, and high bacterial mortality within 48 hours. *Nuc* transcription also varies only 1.5-fold between planktonic and biofilm cells, suggesting that conservative modulation of this activity is critical for proper function. Interestingly, though Nuc and Staphylococcal thermonuclease are similar in size, thermostability, and cation requirements, Nuc is 25-fold more active due to shorter active site loops permitting larger substrates.[75] It has been shown that *Staphylococcal* thermonuclease degrades eDNA as a means to promote biofilm dispersal, thus the

finding of a hyper-degradative nuclease provides a novel tool to combat biofilm persistence.[76] Interspecies facilitation of dispersal has also been observed as a means to prevent competition in which *Bacillus licheniformis* was capable of dispersing established biofilms in the clinically relevant bacteria *E. coli* and *Pseudomonas* via its extracellular DNase NucB within its supernatant.[77] Mechanisms facilitating biofilm dispersal continue to be uncovered, thus elucidating various species approach to eDNA incorporation into biofilms will provide further targets for combating biofilm mediated infections.

Extracellular Biofilm Matrix Protein Dispersion

Biofilm matrix proteins provide one of the most diverse methods by which bacteria may facilitate dispersion through protease digestion or simply detaching adhesion proteins. One of the best understood dispersal mechanisms has been studied in *S. aureus*, where protease activity is implicated in biofilm dispersal.[78] Various proteases have been shown to have differential dispersal magnitudes within *S. aureus* biofilms, with Aur, ScpA, and SspB being some of the most potent biofilm dispersal proteins.[79,80] Protease proteolytic processing activates many proteases, as Aur processes SspA which then processes SspB.[81,82] Protease dispersal in SspA and Aur through the release of cell bound fibronectin binding proteins, degradation of Bap, and Aur-mediated proteolysis of ClfB, in which each of the proteins protolyzed are involved in biofilm formation.[83–85] Cleavage of large adhesins has been observed in *Pseudomonas fluorescens* where cleavage of LapA is mediated by LapG while requiring the α-helical region of LapA after which dispersal is observed.[86,87] A similar

manner of dispersal occurs through cleavage of LapA by LapG in *P. putida*.[88] In *P. aeruginosa* there is no homologue of LapA, however, a large adhesion CdrA is cleaved by its LapG, indicating that *Pseudomonas* utilizes LapG to mediate dispersal through a variety of adhesion proteins.[89]

Though cleavage provides a convenient means to mediate biofilm dispersal, some peptides such as *B. subtilis* TasA fibrils show resistance to proteolysis by native proteases, necessitating another mechanism to facilitate dispersal.[90] To conduct this process, *B. subtilis* employs a racemase to convert L-amino acids to D-amino acids, which subsequently alter association of the fibril scaffold TapA, and facilitate the release of TasA fibers which results in biofilm dispersal.[91,92] Interestingly enough D-amino acids inhibited biofilm formation in *S. aureus* and *P. aeruginosa*, however, due to a lack of TasA in these species, the mechanism of this action is unclear. It has been shown that the presence of D-amino acids did not prevent initial attachment in *S. aureus* but prevented larger assembly without hampering exopolysaccharide production.[93] The molecular mechanism of D-amino acids as dispersal agents requires further investigation that provides an attractive agent to combat biofilm mediated infections.

Matrix Lipid Dispersion

Lipid-mediated or surfactant-mediated biofilm dispersal has become an increasingly attractive field due to discoveries of compounds that inhibit biofilm development or mediate diaspora. Interestingly, some of these discoveries have arisen due to structural similarity to short chain fatty acid signaling molecules. One such

compound *cis*-2-decenoic acid is produced by *P. aeruginosa* and has been shown to disperse the established biofilms of *E. coli, K. pneumoniae, P. mirabilis, S. pyogenes, B. subtilis, S. aureus,* and *C. albicans*.[94] Fatty acids such as glycerol monolaurate have shown antibiofilm activity against *S. aureus* and *H. influenzae*.[95] Recently, palmitoleic and myristoleic fatty acids have gained popularity in combating biofilms in *A. baumannii*.[96] The effectiveness of these fatty acid compounds has been shown to be in part due to its effect on the bacterial quorum sensing system, however, other effects may be imparted when facilitating biofilm dispersal.

Other surfactant mediated dispersal mechanisms include cationic lipopeptides have been identified as bactericidal, with studies of synthetic lipopeptides causing up to a 10,000-fold reduction in *P. aeruginosa*.[97] Other biosurfactants have been produced by bacteria themselves, such as *Bacillus* which was found to produce two antiadhesive surfactants of the fengycin-like family against *S. aureus* and *E. coli* that decreased biofilm formation by up to 97%.[98] Interestingly, another avenue of surfactant mediated dispersal termed phenol-soluble modulins or PSMs has been shown to mediate dispersal of some biofilms, while stabilizing others.[99] In the case of stabilization, fibrilization is observed, however the instigation of transition into fibrils is unknown. In dispersal, PSMs have been demonstrated in *S. epidermidis* where detachment is observed on the outskirts of the biofilm and continuous detachment follows.[100] It will be interesting to see further research into lipid-based dispersal and the role of bacterial produced lipids and biosurfactants in biofilm development and dissemination.

Chapter 2: Biofilm Matrix Proteins Impact on Biofilm Formation

Joseph Kiblen and Fitnat H. Yildiz

Introduction

Vibrio cholerae biofilm formation involves the production of *Vibrio* polysaccharide (VPS) and three secreted matrix proteins RbmA, Bap1, and RbmC.[101102] The 26-kDa RbmA is the most well characterized biofilm matrix protein, as deletion of RbmA causes increased cell-cell distance, fragile biofilm formation, and decreased colony biofilm architecture.[103] RbmA is distributed throughout the biofilm and mediates cell-cell interaction by acting as a scaffold protein between cells and VPS.[101] RbmA's crystal structure revealed two fibronectin type III folds which have been shown to connect two RbmA monomers through a linker segment.[104] Higher order structure formation of RbmA is induced through VPS binding by the second fibronectin-III domain.[105]

The other two matrix proteins Bap1 and RbmC are less understood. Bap1 is comprised of two VCBS domains, a putative calcium binding site, and a Lectin domain. Deletion of the 75-kDa *bap1* results in a substantial defect in colony biofilm architecture and surface adhesion.[106] Bap1 is localized in biofilm envelopes, however, Bap1 is localized most abundantly at the cell-surface interface.

RbmC is comprised of two VCBS domains, a putative calcium binding site, and two Lectin domains. *V. cholerae* strains lacking the 104-kDa RbmC shows little change in colony biofilm architecture, however, without RbmC, matrix retention of VPS was abrogated.[101,107] RbmC is localized at the periphery of the biofilm matrix enveloping microcolonies, however, its interaction with matrix components is unknown.[101] A *V. cholerae* strain with deletion of both Bap1 and RbmC shows a marked decrease in colony biofilm architecture, 3D biofilm architecture, and surface

adhesion, suggesting these proteins have a partially redundant role in biofilm formation. RbmC is localized in biofilm envelopes, however, while Bap1 shares localization in biofilm envelope, it is most abundant at the cell-surface interface.[101] Importantly, under dynamic flow conditions, the single deletion of *bap1* or *rbmC* results in little defect of mature biofilm formation, whereas the deletion of *bap1* and *rbmC* results in a strain that is unable to maintain adherence indicating that matrix proteins are required for biofilm formation, however, their specific role is unknown. The overlap between Bap1 and RbmC domain ontology and partially similar biofilm localization motivated a better understanding of these protein's role in biofilm formation.

V. cholerae biofilms contain extracellular proteases that may influence biofilm protein stability thus impacting biofilm architecture. One such protease, the hemagglutinin protease or HapA, constitutes the majority of the matrix proteases and is the primary protease responsible for degradation of the matrix proteins RbmA and GbpA.[108,109110] Two other protease PrtV and IvaP are thought to act as accessory protease for RbmA proteolysis.[108] PrtV is found distributed within the biofilm matrix and in membrane vesicles where it is also involved in GbpA proteolysis.[109,111] IvaP is an extracellular protease, however, a N-terminal I9 protease inhibitor domain temporarily prevents auto-proteolytic processing, where processed IvaP can inhibit intelectin binding to *V. cholerae* in the host environment.[112] Protease targets found during host colonization demonstrate their breadth of use as both regulatory and pathogenic proteins.[113,114]

Here we report our results to demonstrate that individual VCBS and Lectin domains within Bap1 and RbmC contribute to biofilm architecture. We found that VCBS domains were sufficient to improve biofilm compaction, organization. and surface adhesion. We then explored protein interactions to provide a mechanism for Bap1 and RbmC contributions to biofilm formation. We determined that Bap1 and RbmC both interact with RbmA. We subsequently show that Bap1 but not RbmC interacts with VPS. We then show that of Bap1 and RbmC Lectin domain mutations impact biofilm formation. We then determined the Bap1 and RbmC post-translational proteolytic regulation by the HapA protease.

Experimental Procedures

Matrix Protein Domain Bioinformatics

Multiple programs were used in order to best define boundaries including the Conserved Domain Database (CDD), InterPro Consortium, and PHMMER project. Each of the domain boundaries can be seen in *Figure* 2.2, where the PHMMER residues were chosen for the project. Briefly, for each protein, the sequences in FASTA format were uploaded to each tool. Sequence conservation was conducted through PRALINE multiple sequence alignment program as seen in *Figure* 2.2 where four separate comparisons were conducted.

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in table 1. The *V. cholerae* rugose variant was used as a parent strain (Yildiz and Schoolnik, 1999). Mutants were generated in the rugose genetic background. *V. cholerae* and *Escherichia coli* strains were grown aerobically, at 30°C and 37°C, respectively, unless otherwise noted. Cultures were grown in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl), pH 7.5, unless otherwise noted. LB agar medium contains 1.5% (w/v) granulated agar (BD Biosciences, Franklin Lakes, NJ). Concentrations of antibiotics and inducer used, when appropriate, were as follows: ampicillin (Amp), 100 μg/mL; rifampicin (Rif), 100 μg/mL; gentamicin (Gm), 50 μg/mL and isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.1 to 1.0 mM. In-frame deletion, point mutation and GFP-tagged strains were generated according to protocols previously published (Fong et al., 2006; Fong et al., 2010; Giglio et al., 2013). *V. cholerae* strains harboring in-frame chromosomal *rbmA*-Myc, *rbmC*-3×FLAG and *bap1*-3×HA truncated versions were generated by allele exchange between the chromosomal copy of the genes and the truncated or tagged versions of the genes on the suicide plasmid pGP704*SacB*28, according to previous published protocol.[11] Strains containing wild-type *rbmC*-3×FLAG and *bap1*3×HA used to generate truncated constructs are shown in Table 1 strains and plasmids.

Recombinant DNA techniques

DNA manipulations were carried out by standard molecular techniques according to manufacturer's instructions. Gibson Assembly master mix, restriction

and DNA modification enzymes were purchased from New England Biolabs (NEB, Ipswich, MA). Polymerase chain reactions (PCR) were carried out using primers purchased from Integrated DNA Technologies (IDT, San Diego, CA) and the Phusion High-Fidelity PCR kit (NEB, Ipswich, MA), unless otherwise noted. Primers used in the present study are listed in Supplementary file 1. Constructs were verified by DNA sequencing (UC Berkeley DNA Sequencing Facility, Berkeley, CA).

Western Blot Analysis

Total proteins extracted from whole-cell (WC, 100 μg) or precipitated from culture supernatant (CS, 30 μg)) were separated on 12% SDS-PAGE and transferred to PVDF for Western blot analysis, following previously described protocol (Giglio et al., 2013). For planktonic cells, overnight cultures were diluted 1:200 and grown at 30°C in LB medium with shaking at 200 rpm until Od_{600}: 0.3-0.4 unless otherwise indicated. Culture supernatant was separated from the cells by centrifugation at 5000 x *g*. Whole-cell samples were prepared by resuspending the cell pellets in 2% (w/v) SDS in SIGMA*FAST* Protease Inhibitor Tablets (PROD: S8820-20TAB). The culture supernatant fractions were collected and bovine serum albumin (BSA) (1 mg) was added to 30 mL of each culture supernatant fractions as an additional loading control. Total protein in the culture supernatant was precipitated with 13% (v/v) trichloroacetic acid (TCA) at 4°C overnight, followed by centrifugation at 45,000 x *g* for 1 hr. The protein pellets from the culture supernatant were washed with 7 mL ice-cold acetone and resuspended in 1x PBS and SIGMA*FAST* Protease Inhibitor Tablets. Protein concentrations were estimated using a Pierce BCA protein assay kit

(Thermo Fisher Scientific, Waltham, MA) and BSA as standard. Detection of proteins used anti-FLAG polyclonal mouse antibody at a 1:1000 dilution and anti-HA polyclonal rabbit antibody at a 1:1000 dilution. For detection of VPS, 3 μL of serially diluted extract (1:1-6250) was spotted onto nitrocellulose membranes and immunoblot analyses were carried out using anti-VPS antiserum at a dilution of 1:1000, and goat anti-rabbit horseradish-peroxidase-conjugated antibody. Additional loading controls where appropriate were used, and 1:10000-diluted monoclonal mouse anti-RNAP (BioLegend Neoclone, San Diego, CA). Secondary goat anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Paso Robles, CA) was used at a dilution of 1:2000. The SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and a Bio-Rad ChemiDoc MP imaging system were used for detection and capturing of the Western blot signals. Western blot analysis was carried out with two biological replicates.

Analysis of Biofilm formation

For analysis of spot biofilm morphology (corrugation development), cultures were grown overnight at 30 ºC shaking, then 3 μL of 1:200 diluted culture were plated onto 20 ml LB agar plates unless otherwise stated. Samples were then incubated at 30ºC for 48 followed by 25ºC for 48 hours unless otherwise stated. Imaging of spot biofilms were each sized to 1 mm unless otherwise stated.
For flow-cell biofilm studies, Ibidi μ-Slide VI0.4 (Ibidi 80601, Ibidi LLC, Verona, WI) flow-cell chambers were inoculated with 180 μL of overnight-grown cultures, normalized to an OD_{600} of 0.02. Flow-cell chambers were incubated at room

temperature for 1 hr, then flow of diluted LB (0.2 g/L tryptone, 0.1 g/L yeast extract, 10 g/L NaCl, pH 7.5) was initiated at a rate of approximately 45 mL/h and continued for up to 24 hr. Confocal images were obtained on a Zeiss LSM 880 laser scanning confocal microscope (Zeiss, Dublin, CA). Images were obtained with a 40 and 10 dry objective and were processed using Imaris software (Biplane, South Windsor, CT). Confocal laser scanning microscopy (CLSM) were carried out with at least two biological replicates. Colony forming units (CFU) of the inoculated culture for flow-cells in the CLSM experiments were quantified by dilution plating and were repeated with at least two biological and technical replicates.

For static biofilm studies cultures were grown overnight at 30 ºC shaking, then 1 mL of 1:200 diluted culture into LB was inoculated into 2 well glass bottom coverslips (Ibidi 80287) with or without 2.5 μM matrix proteins and grown for 8 hours at 30 ºC unless otherwise stated. 800 μL of LB was then gently removed and biofilms were washed twice with PBS (400 μL). Samples were then imaged and processed as done for flow biofilms.

Protein Purification

For protein purification, recombinant *E. coli* BL21 (DE3) cells carrying expression plasmids were grown at 37°C in LB medium until optical density at 600 nm (OD_{600}) reached 0.6 to 0.8. Induction was carried out by adding IPTG to a final concentration of 0.5 mM and the cultures were grown overnight (16 to 18 hr) at 18°C. Cells were harvested by centrifugation and lysed in their associated buffer shown in table 2 and SIGMA*FAST* Protease Inhibitor Tablets via three passes of cell

disruption. Proteins were purified using glutathione sepharose resin via gravity flow column chromatography. The column was first washed with 200-300 mL of the proteins associated buffer. Elution was carried out with each protein's associated buffer and 20 mM Glutathione pH-8. Buffer exchange was carried out with the eluted protein and dialysis. For cleaved proteins 4 mg of His-TEV was added to the elution and cleavage was conducted overnight at 4 ℃ in the proteins buffer and 0.5 mM DTT followed by another 24-hour dialysis at 4 ℃ without DTT to prepare for Nickel chromatography. After proteolysis, cleaved tag and the protease were removed by another round of chromatography using gravity flow IMAC with Nickel resin chromatography. Further purified proteins used size-exclusion chromatography on Superdex 200 10/300 (GE Healthcare Bio-Sciences, Marlborough, MA). Protein was concentrated using Amicon Ultra-15 centrifugal filter units and quantified using Pierce BCA.

VPS Purification

VPS Extraction was conducted using a protocol very similar to (Fong, 2010; Yildiz, 2014). Briefly, 500 µL overnight-grown cultures were spread on cellulose dialysis membranes placed on the surface of (10) 50 mL LB agar plates. Overnight-grown biofilms on LB agar cellulose were then harvested and resuspended into 30 mL of PBS on ice and incubated for 24 hours at 4 ℃. Normalization was carried out by adjusting each culture to the same OD_{600}. Biofilm cells were then collected by centrifugation at 5,000xg, 4°C, 45 min. The supernatant was then clarified with additional centrifugation at 8,000×g, 4°C, 45 min. The crude VPS pellet was

resuspended in nuclease buffer (40 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% NaN_3. Next, 2 units/mL of DNase I and .25 units/mL of RNase A were added to the VPS suspension, followed by incubation at 37 °C shaking for 3 hr. Proteinase K was then added at a final concentration of 200 µg/mL, followed by shaking overnight at 37 °C. Phenol/chloroform extractions (equal volumes) were carried out, followed by precipitation with 3 volumes of 100% ethanol at -20ºC. VPS was then pelleted twice by centrifugation at 20,000xg for 20 min at 4ºC, intermittently resuspended with 70% ethanol. Pellets were then air-dried and resuspended in 100-200 µl water containing 0.05% NaN_3. Stored short-term at 4ºC or long-term at -20ºC. For purity analysis, 3 µL of serial diluted VPS (1-1/6250) was spotted onto nitrocellulose membranes and immunoblot analyses were carried out using rabbit αVPS antisera and imaged as termed in Western Blot Analysis.

Co-Immunoprecipitation

To assay for interaction between Bap1 and RbmC with RbmA the manufacturer's protocol was used. Briefly, *V. cholerae* cells containing chromosomal Myc-RbmA, HA-Bap1, and FLAG-RbmC were grown overnight followed by a 1:200 dilution grown into fresh LB. The cells were spun down, resuspended in protease inhibitor (S8830-20TAB) in 200 mM NaCl and 35 mM, lysed, then the soluble fraction was collected for incubation with protein G beads. 50 µL of Invitrogen Protein G Beads I (10003D) was incubated with 8 µg of either αMyc, αHA, αFLAG antibodies for 1 hr at RT on a low-speed rotator. Followed by the addition of either the whole cell or culture supernatant extracts for 1 hour on a low-speed rotator. Beads

were then washed three times with PBS plus 0.02% Tween-20. Proteins were eluted by resuspension in loading buffer and incubated at 80 ℃ for 10 minutes. Immunoblot analysis of samples was conducted as above.

Bap1and RbmC interaction with VPS was conducted similarly, manufacturer's protocol was used. Briefly: *V. cholerae* cells containing chromosomal Myc-RbmA, HA-Bap1, and FLAG-RbmC were grown overnight followed by a 1:200 dilution grown into fresh LB. The cells were spun down, resuspended in protease inhibitor (S8830-20TAB) and 200 mM NaCl and 35 mM, lysed, then the soluble fraction was collected for incubation with protein G beads. 50 μL of Invitrogen Protein G Beads I (10003D) incubated with 8 μg of the αVPS, antibody for 1 hr at RT on a low-speed rotator. Followed by the addition of 50 μL of purified VPS at a 1:10 dilution to 500 μL of PBS and incubation with the beads for 1 hr at RT on a low-speed rotator. The wash and elution were identical to the protein immunoprecipitation conditions.

Table 2.1

***E. coli* strains**	**Relevant Genotype**	**Source**
S17-1λ*pir*	Tpr Smr *recA thi pro* rK- mK+ RP4::2-Tc::MuKm Tn*7*λ*pir*	(de Lorenzo et al., 1994)
DH5α-1λpir	F- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*) *U169* (ϕ80d*lac*Δ*M15*)	Promega
BL21(DE3)	F- *ompT hsdSB* (rB-mB-) *gal dcm* (DE3)	Invitrogen
***V. cholerae* strains**		
FY_Vc_2	*Vibrio cholerae* O1 El Tor A1552, wild type rugose, Rif^r	Yildiz & Schoolnik (1999)
FY_Vc_240	Rugose-*gfp*, V. cholerae O1 El Tor A1552, wild type rugose, Rif^r Gm^r	Beyhan & Yildiz (2007)
FY_Vc_686	RΔ*rbmC*, FY_Vc_2 Δ*rbmC*, Rif^r	Fong & Yildiz (2007)
FY_Vc_1367	RΔ*bap1*, FY_Vc_2 Δ*bap1*, Rif^r	Fong & Yildiz (2007)
FY_Vc_14323	R-Bap1 1-316-3XHA, Rif^r	This study
FY_Vc_1432	R-Bap1 1-443-3XHA, Rif^r	This study
FY_Vc_13378	R-Bap1_D348A-3XHA, Rif^r	Fong & Yildiz (2007)
FY_Vc_13364	R-Bap1_Y385A-3XHA, Rif^r	This study
FY_Vc_13432	R-Bap1_W387A-3XHA, Rif^r	This study
FY_Vc_1400	RΔ*rbmC* Δ*bap1*, FY_Vc_1367 Δ*rbmC*, Rif^r	Fong & Yildiz (2007)
FY_Vc_15221	RΔ*rbmC*, Bap1 1-316-3XHA, Rif^r	This study

FY_Vc_15222	R∆*rbmC*, Bap1 1-443-3XHA, Rif^r	This study
FY_Vc_14314	R∆*bap1*, RbmC 1-485-3XFLAG, Rif^r	This study
FY_Vc_14316	R∆*bap1*, RbmC 1-650-3XFLAG, Rif^r	This study
FY_Vc_14315	R∆*bap1*, RbmC 1-796-3XFLAG, Rif^r	This study
FY_Vc_13435	∆*bap1,* rbmC D539A-3XFLAG, Rif^r	This study
FY_Vc_13080	∆*bap1,* rbmC Y575A-3XFLAG, Rif^r	This study
FY_Vc_13083	∆*bap1,* rbmC W577A-3XFLAG, Rif^r	This study
FY_Vc_13283	∆*bap1,* rbmC D853A-3XFLAG, Rif^r	This study
FY_Vc_13286	∆*bap1,* rbmC T870A-3XFLAG, Rif^r	This study
FY_Vc_13489	∆*bap1,* rbmC N871A-3XFLAG, Rif^r	This study
FY_Vc_13429	∆*bap1,* rbmC Y894A-3XFLAG, Rif^r	This study
FY_Vc_13426	∆*bap1,* rbmC W896A-3XFLAG, Rif^r	This study
FY_Vc_8092	R∆*ctxAB* ∆*bap1 rbmA*-myc *rbmC*-3xFLAG, Rif^r	Berk et al., (2012)
FY_Vc_8094	R∆*ctxAB* ∆*rbmC rbmA*-myc *bap1*-3xHA, Rif^r	Berk et al., (2012)
FY_Vc_9441	R∆*bap1*∆*rbmC RbmA*-Myc ∆*ctxAB*, Rif^r	Yildiz (unpublished)
FY_Vc_1321	FY_Vc_686 mTn*7*-GFP, Rif^r, Gm^r	Fong & Yildiz (2007)
FY_Vc_1392	FY_Vc_1367 mTn*7*-GFP, Rif^r, Gm^r	Fong & Yildiz (2007)

FY_Vc_15223	RΔ*rbmC* mTn7-gfp, Bap1 1-316-3XHA, Rif^r, Gm^r	This study
FY_Vc_15224	RΔ*rbmC* mTn7-gfp, Bap1 1-443-3XHA, Rif^r, Gm^r	This study
FY_Vc_14319	RΔ*bap1* mTn7-gfp, RbmC 1-485-3XFLAG, Rif^r, Gm^r	This study
FY_Vc_14320	RΔ*bap1* mTn7-gfp, RbmC 1-650-3XFLAG, Rif^r, Gm^r	This study
FY_Vc_14321	RΔ*bap1* mTn7-gfp, RbmC 1-796-3XFLAG, Rif^r, Gm^r	This study
FY_Vc_4327	RΔ*vps*-IΔ*vps*-II, rugose variant with deletion of *vpsA-K* and *vpsL-Q*, Rif^r	Fong & Yildiz (2010)
FY_Vc_4329	RΔ*rbmA*Δ*rbmC*Δ*bap1*Δ*ctxAB*, Rif^r	Berk et al., (2012)
FY_Vc_6431	Rugose *rbmA*-Myc, *bap1*-3xHA, *rbmC*-3xFLAG, Rif^r	Berk et al., (2012)
FY_Vc_10559	Rugose Δ*rbmA*Δ*bap1*Δ*rbmC* Δ*vps-opI//opII*, Rif^r	Yildiz (unpublished)
FY_Vc_11808	RΔ*ctxAB* r*bmA*-Myc, r*bmC*-3xFLAG, *bap1*-3xHA Δ*hapA*, Rif^r	This study
FY_Vc_9935	RΔ*hapA, FY_Vc_2 ,* Rif^r	Yildiz (unpublished)
FY_Vc_9947	RΔ*prtV*, FY_Vc_2 , Rif^r	Yildiz (unpublished)
FY_Vc_10283	RΔ*hapA*Δ*prtV*Δ*IvaP*, Rif^r	Fong et al., (2017)
Plasmids		
pUX-BF13	oriR6K helper plasmid, mob/oriT, provides the Tn7 transposition function in trans, Ap^r	G. Schoolnik
pMCM11	pGP704::mTn7-*gfp*, Gm^r Ap^r, Mini-Tn7 vector harboring a constitutively expressed gfp cassette	M. Miller/G. Schoolnik

pGP704*sacB*28	pGP704 derivative, *mob*/*oriT sacB*, Ap^r	G. Schoolnik
pFY-5450	pGP704sac28- Bap1 D348A-HA tagged, Ap^r	This study
pFY-5451	pGP704sac28- Bap1 Y385A-HA tagged, Ap^r	This study
pFY-5452	pGP704sac28-Bap1 W387A-HA tagged, Ap^r	This study
pFY-5453	pGP704sac28- RbmC -D539A-3xFLAG, Ap^r	This study
pFY-5454	pGP704sac28- RbmC -Y575A-3xFLAG, Ap^r	This study
pFY-5455	pGP704sac28- RbmC -W577A-3xFLAG, Ap^r	This study
pFY-5456	pGP704sac28- RbmC -D853A-3xFLAG, Ap^r	This study
pFY-5457	pGP704sac28- RbmC -T870A-3xFLAG, Ap^r	This study
pFY-5458	pGP704sac28- RbmC -Y894A-3xFLAG, Ap^r	This study
pFY-5459	pGP704sac28- RbmC -W896A-3xFLAG, Ap^r	This study
pFY-5713	pGP704sacB28 RbmC 1-485-3XFLAG, Ap^r	This study
pFY-5715	pGP704sacB28 RbmC 1-650-3XFLAG, Ap^r	This study
pFY-5714	pGP704sacB28 RbmC 1-796-3XFLAG, Ap^r	This study
pFY-5995	pGP704sacB28 Bap1 1-316-3XHA, Ap^r	This study
pFY-5996	pGP704sacB28 Bap1 1-443-3XHA, Ap^r	This study
pFY-3509	pGP704sac28-Δ*hapA*, Ap^r	Fong et al., (2017)

pHisGST	IPTG-inducible vector for expression of recombinant proteins with N-terminal His6-GST tags, cleavable by TEV protease, Ap^r,	(Xu et al., 2015)
pFY-3552	pHisGST-Bap1-VCBS-2, Ap^r	This study
pFY-2161	pHisGST-RbmC-Lectin-2 #B1, Apr	This study
p2GT	IPTG-inducible vector with TEV-cleavable N-terminal His6-GST fusion tag.	Addgene #29707 (This study)
pFY-5707	p2GT RbmC 164-485, Ap^r	This study

Table 2.2

Resuspension Buffer	Composition
1	300 mM NaCl, 50 mM Tris pH = 8
2	200 mM NaCl, 35 mM Tris pH = 8

Protein	Resuspension Buffer
His-GST- RbmC VCBS-1	1
His-GST- RbmC Lectin-2	1
His-GST- Bap1 VCBS-2	2
Vibrio Matrix Tagged - Cell lysate	2

Results

Domain organization of *V. cholerae* biofilm matrix proteins Bap1 and RbmC.

To better understand how matrix protein domain organization controls their impact on biofilm formation, we first needed to establish a reference point for what differences could be involved. In *Figure 2.1* the domain organization of Bap1 and RbmC is displayed. Bap1 contains two VCBS domains and one Lectin domain. RbmC contains two VCBS domains and two Lectin domains. Because of the difference in domain number, understanding the impact of each domain would allow us to further determine their impact in each protein.

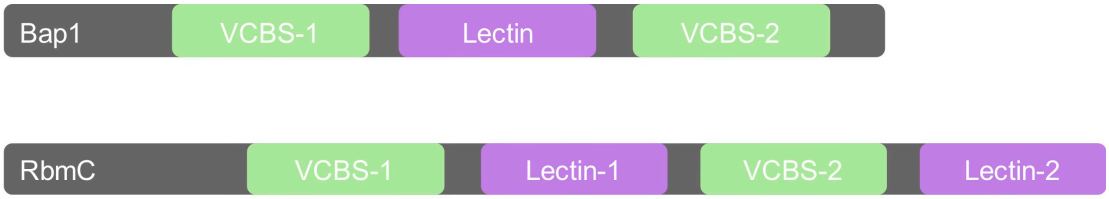

Bap1
VCBS-1
Lectin
VCBS-2
RbmC
VCBS-1
Lectin-1
VCBS-2
Lectin-2

Figure 2.1. Domain organization of *V. cholerae* biofilm matrix proteins Bap1 and RbmC. Bap1 and RbmC both harbor VCBS repeats or *Vibrio-Colwellia-Bradyrhizobium-Shewanella* repeats, and Lectin carbohydrate binding domains.

Domain organization of *V. cholerae* biofilm matrix proteins Bap1 and RbmC

To analyze the differences between domains in Bap1 and RbmC, determining the most representative domain boundaries was necessary. We used three separate domain prediction tools to determine N- and C-terminal domain boundaries. The HMMER modeling was then selected for our domain boundaries due to the strength of prediction and is listed in *Figure 2.2*. To assess differences in sequence conservation between VCBS and Lectin domains, the sequences were submitted as Bap1 VCBS domains or RbmC VCBS domains individually, then all four VCBS domains in one alignment. Lectin domains were analyzed together, as Bap1 contains a single Lectin domain.

	Bap1-VCBS-1	Bap1-VCBS-2	RbmC-VCBS-1	RbmC-VCBS-2
HMMER	125-185	519-571	317-377	689-746
CDD	125-185	523-571	317-377	689-746
Inter-Pro	58-295	528-665	252-491	590-794

	Bap1-Lectin	RbmC-Lectin-1	RbmC-Lectin-2
HMMER	317-452	507-637	822-956
CDD	318-451	508-637	823-956
Inter-Pro	317-450	508-637	822-955

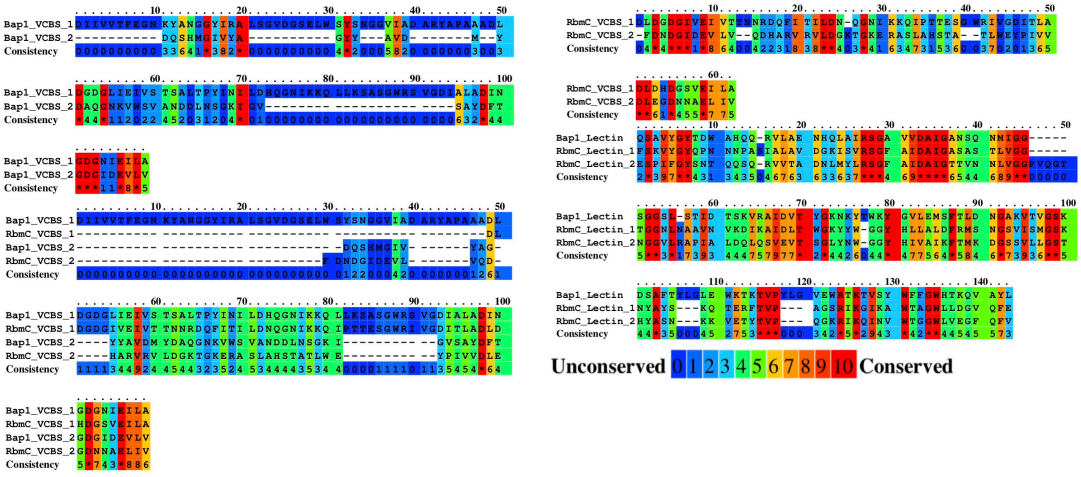


Figure 2.2. Matrix proteins Bap1 and RbmC display a high-degree of interdomain residue conservation. Matrix proteins sequences were evaluated by three separate domain prediction tools; HMMER, the Conserved Domains Database CDD, and InterPro to identify domain residue boundaries. The HMMR boundaries of VCBS and Lectin domains were then uploaded to the Praline informatics tool to assess sequence conservation.

Matrix protein constructs of Bap1 and RbmC harboring successive domain deletions

To determine the contribution of the matrix protein domains, we evaluated the impact of each domain in Bap1 and RbmC. To accomplish this goal, we deleted successive portions of Bap1 and RbmC and determined consequences of domain depletion by a set biofilm phenotypic analysis tools. The constructs were then generated chromosomally under their native promotor which can be seen in *Figure 2.3*. Prior to testing their impact on biofilm formation, we checked to ensure that these constructs are expressed as seen in the western blot in *Figure 2.4*. Both Bap1 and RbmC protein constructs are expressed, however, the level of expression of the Bap1 constructs is less than wild-type Bap1. The confirmed expression of both constructs allowed us to characterize their impact.


Bap1
VCBS-1
Lectin
VCBS-2
HA
B-1
VCBS-1
HA
B-2
VCBS-1
Lectin
HA
RbmC
VCBS-1
Lectin-1
VCBS-2
Lectin-2
FLAG
C-1
VCBS-1
FLAG
C-2
VCBS-1
Lectin-1
FLAG
C-3
VCBS-1
Lectin-1
VCBS-2
FLAG

Figure 2.3. Matrix protein constructs of Bap1 and RbmC harboring successive domain deletions. Portions of Bap1 and RbmC containing a VCBS or Lectin domain were deleted additively from Bap1 and RbmC harboring a HA or FLAG epitope tag.

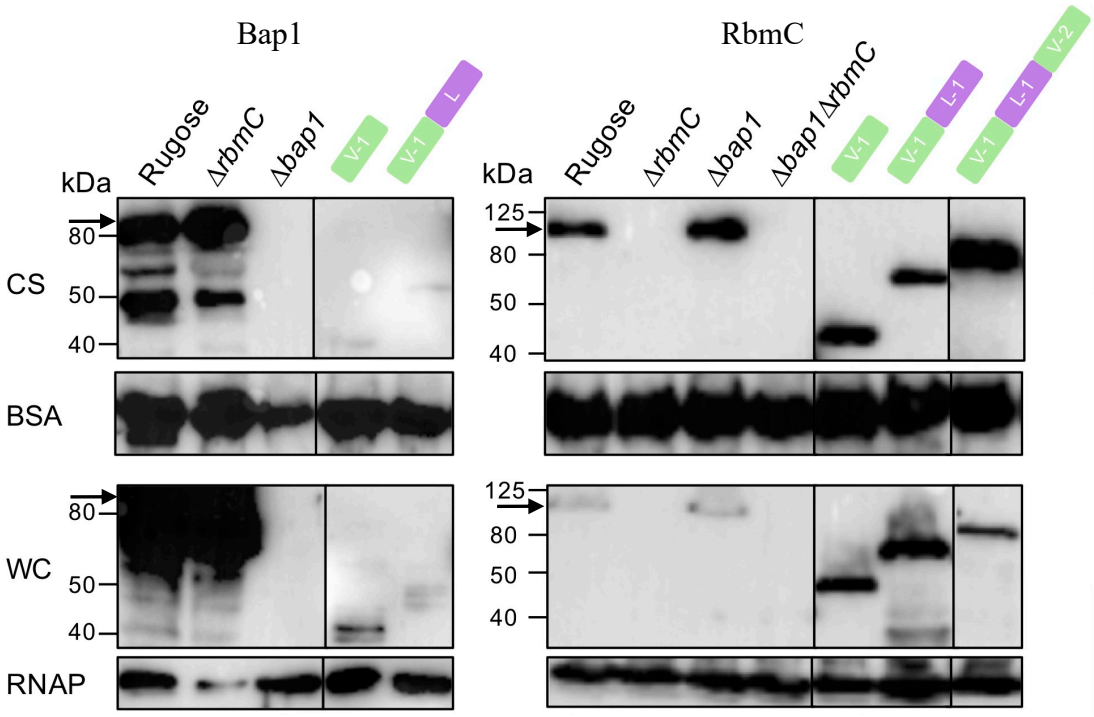
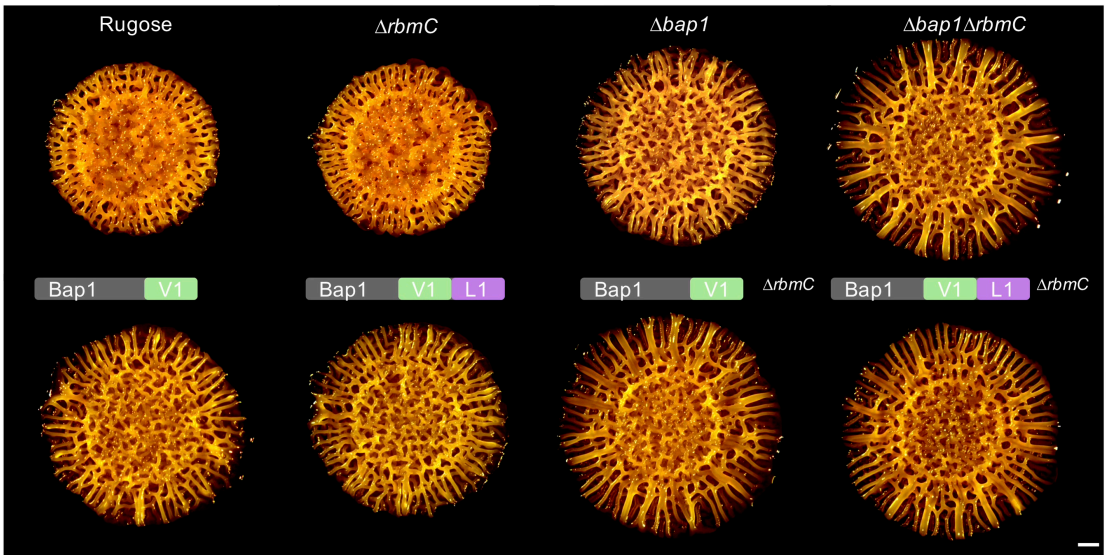

Bap1
RbmC
Rugose
ΔrbmC
Δbap1
Δbap1ΔrbmC
V-1
L
L-1
V-2
kDa
125
80
50
40
CS
BSA
WC
RNAP

Figure 2.4. Evaluation of Bap1 and RbmC successive deletion constructs expression. Matrix protein constructs were grown planktonically until early log phase. Whole cell lysate (WC) was resuspended, and the Cell supernatant (CS) was TCA precipitated overnight. 100 µg of the whole cell and 30 µg of precipitated cell supernatant were loaded into a gel for western blot analysis of Bap1 (81 kDa), RbmC (104 kDa), and each successive deletion.

Bap1 and RbmC VCBS and Lectin domains contribute to biofilm formation

Bap1 and RbmC successive deletions were initially analyzed using spot biofilms on agar (*Figure 2.5*). The differences seen appear more distinct radially, as the outer corrugation pattern extends further. This appears to be the case for the each RbmC construct. Bap1 constructs containing the Lectin domain appear more compact than their parent strain (lacking both RbmC and Bap1), though the presence of the first VCBS domain does appear to improve the radial corrugation pattern. To understand the how having these domain constructs contributes to biofilm organization (cell-cell distance, organization) in greater detail, we utilized scanning electron microscopy to characterize the strains harboring RbmC successive deletion constructs biofilm formation (*Figure 2.6*).


Rugose
ΔrbmC
Δbap1
Δbap1ΔrbmC
Bap1 V1
Bap1 V1 L1
Bap1 V1 ΔrbmC
Bap1 V1 L1 ΔrbmC

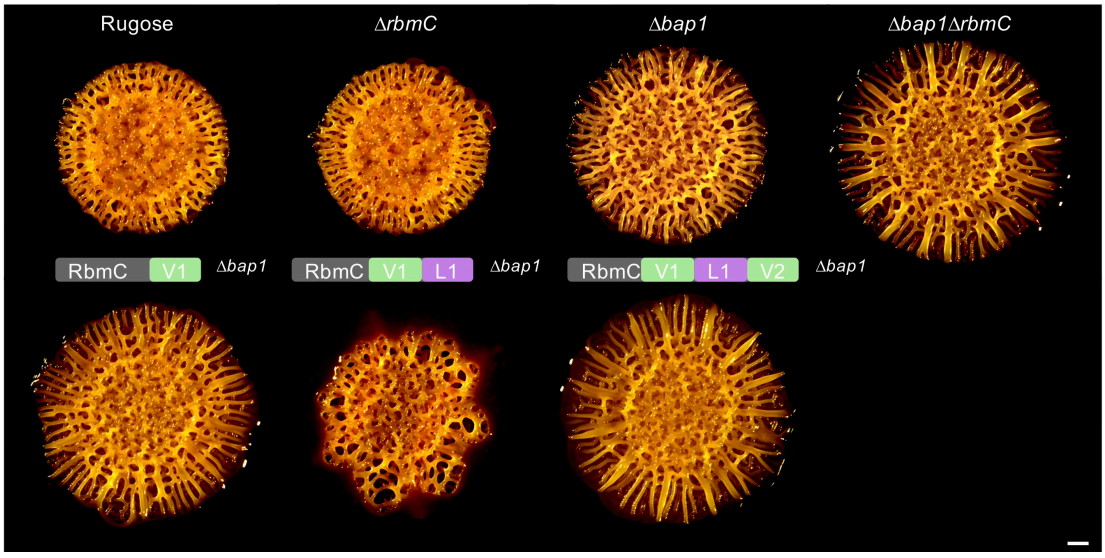

Rugose
ΔrbmC
Δbap1
Δbap1ΔrbmC
RbmC V1 Δbap1
RbmC V1 L1 Δbap1
RbmC V1 L1 V2 Δbap1

Figure 2.5. Bap1 and RbmC VCBS and Lectin domains contribution to biofilm formation. The spot colony morphologies of the strains harboring successive deletions for Bap1 (top) and RbmC (bottom). Each spot consists of 3μL of *V. cholerae* overnight culture (1:200) plated in triplicate on 20 ml LB agar plates for 48 hr at 30°C followed by 48 hr at 25°C.

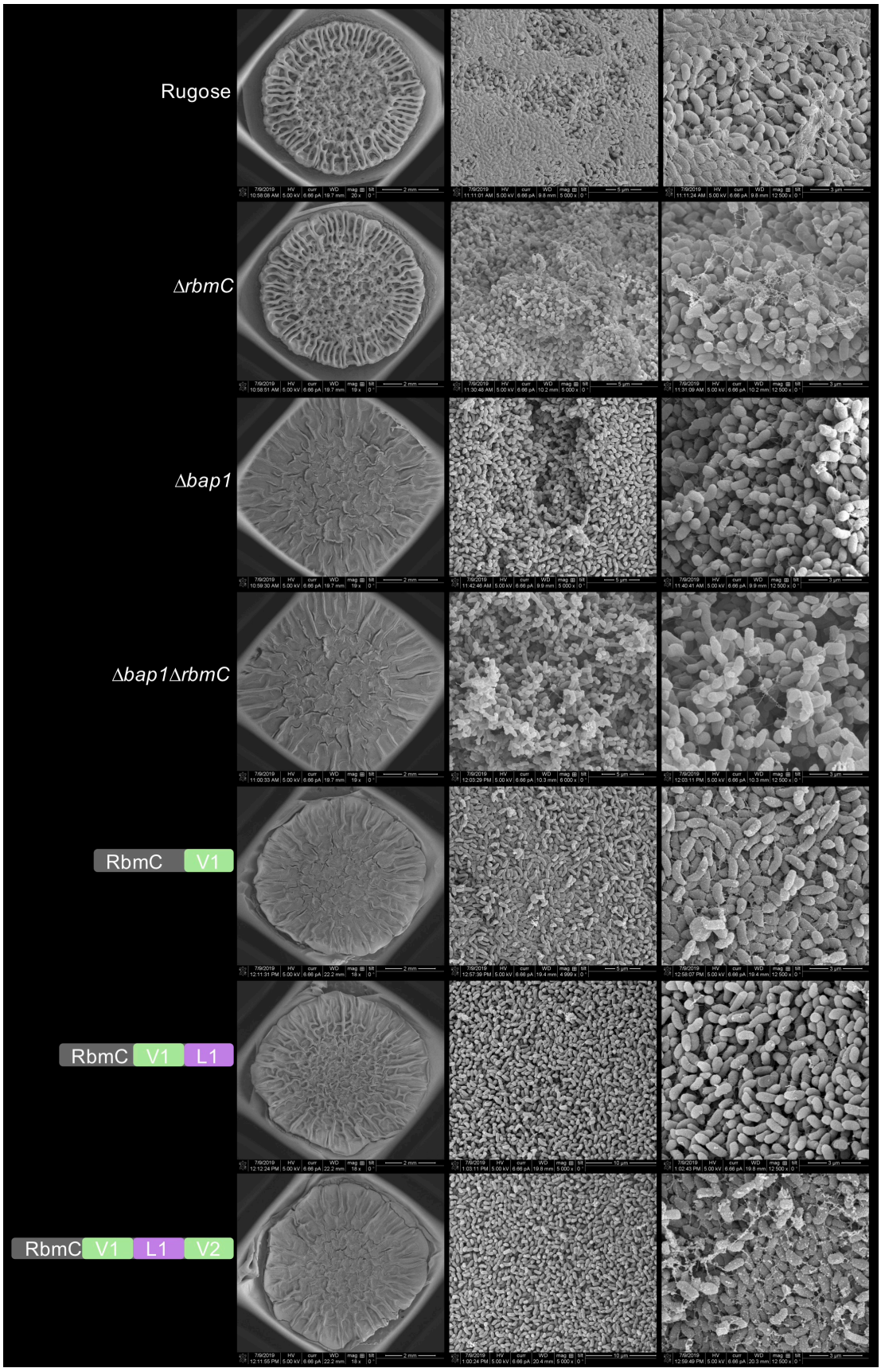
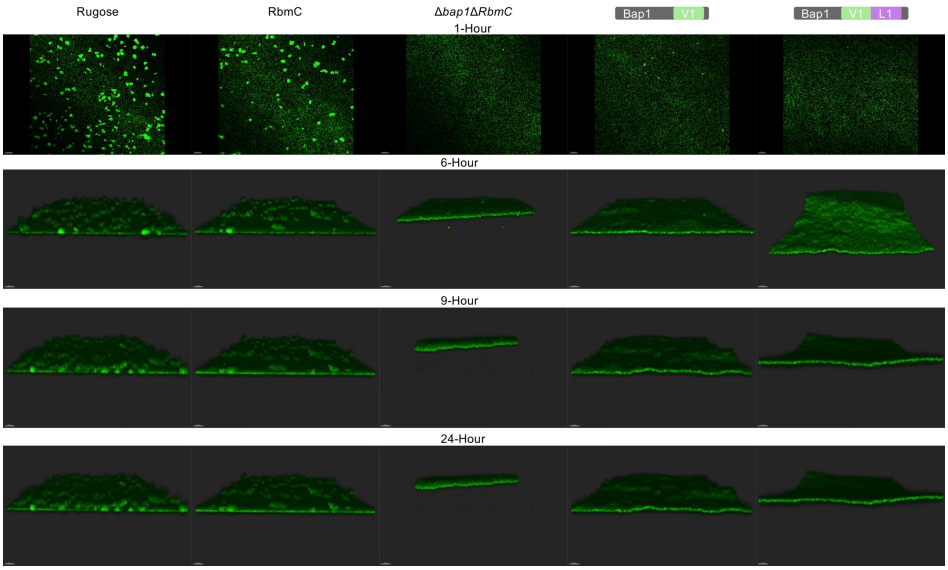

Rugose
ΔrbmC
Δbap1
Δbap1ΔrbmC
RbmC V1
RbmC V1 L1
RbmC V1 L1 V2

Figure 2.6. Visualization of spot biofilms via scanning election microscopy. Spot biofilms were grown using 3 µL of *V. cholerae* overnight culture was plated on 20 ml LB agar plates for 48 hr at 30°C followed by 48 hr at 25°C. Agar spots were excised surrounding spot biofilms, fixed with 2.5% glutaraldehyde, and sequentially dehydrated with increasing concentrations of ethanol until reaching 100%. Magnifications for images of each strain represent <20x, 5000-6000x, and 12,500x.

Bap1 and RbmC domain contributions to biofilm formation under dynamic conditions

Due to the differences in biofilm morphology in the presence of matrix protein domains, we then asked whether this morphological difference would translate into changes in surface adhesion. We analyzed biofilm formation in a flow cell system as a function of time. The results presented in in *Figure* 2.7 show that surface adherence and retention on the surface of the strains with Bap1 and RbmC deletion constructs is increased relative to that of the Δ*bap1*Δ*rbmC* parent strain. The strains containing the first VCBS domain alone appeared to maintain greater adherence when compared to the strains harboring VCBS and Lectin domains. These results suggest that matrix protein domain interactions impact attachment to surfaces.


Rugose
RbmC
Δbap1ΔRbmC
Bap1 V1
Bap1 V1 L1
1-Hour
6-Hour
9-Hour
24-Hour

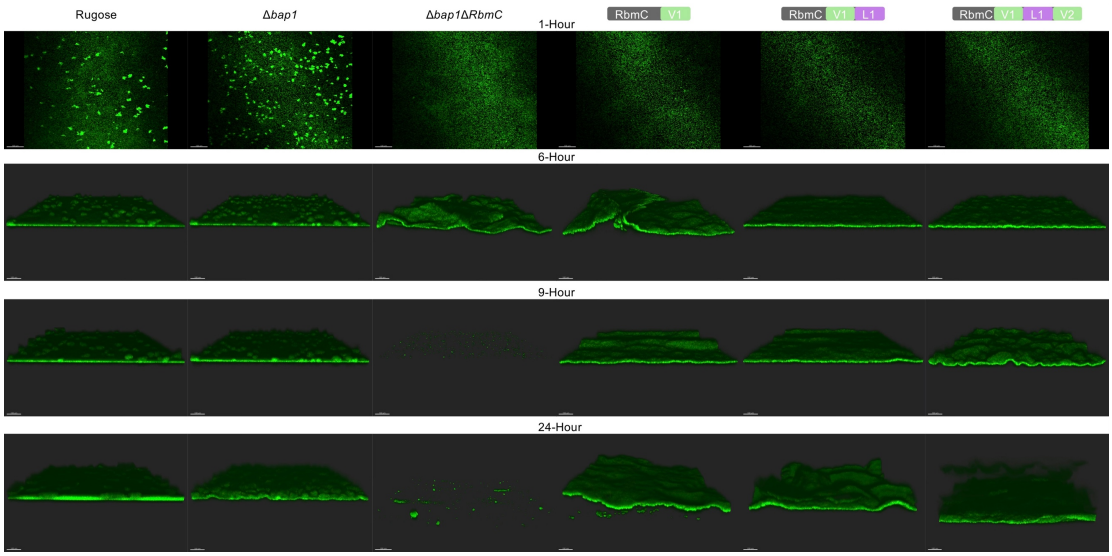
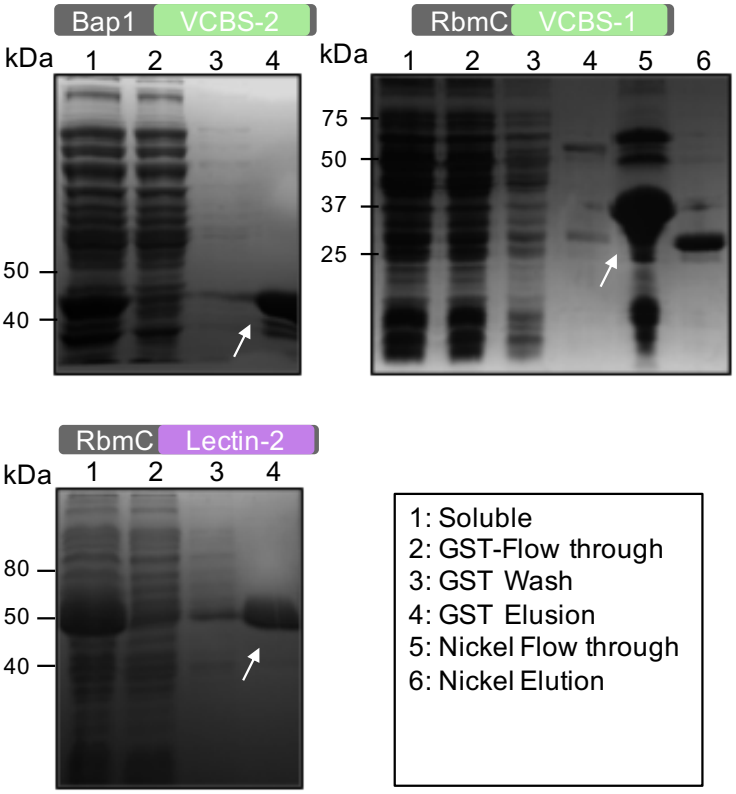

Rugose
Δbap1
Δbap1ΔRbmC
RbmC V1
RbmC V1 L1
RbmC V1 L1 V2
1-Hour
6-Hour
9-Hour
24-Hour

Figure 2.7. Bap1 and RbmC domain contributions under dynamic biofilm conditions. Flow cell biofilms of Bap1 (top) and RbmC (bottom) mutants as shown at 1, 6, 9, and 24 hr post flow at 45 mL/hr. Images were captured using the 488 nm wavelength GFP channel and processed with Imaris are shown.

Purification and addition of VCBS and Lectin domains partially restores biofilm formation to Δ*bap1*Δ*rbmC* biofilms

Due to the observed increase in biofilm adherence that was dependent on VCBS and Lectin domains, we next wondered whether the addition of an isolated domain to a growing biofilm could induce biofilm interactions and facilitate development of biofilm architecture. The results shown in *Figure* 2.8 suggest that addition of isolated VCBS and Lectin domains to a Δ*bap1*Δ*rbmC* strain at 2.5 μM results in improved biofilm formation after 8 hours of static growth.


Bap1 VCBS-2
kDa 1 2 3 4
50
40
RbmC VCBS-1
kDa 1 2 3 4 5 6
75
50
37
25
RbmC Lectin-2
kDa 1 2 3 4
80
50
40
1: Soluble
2: GST-Flow through
3: GST Wash
4: GST Elusion
5: Nickel Flow through
6: Nickel Elution

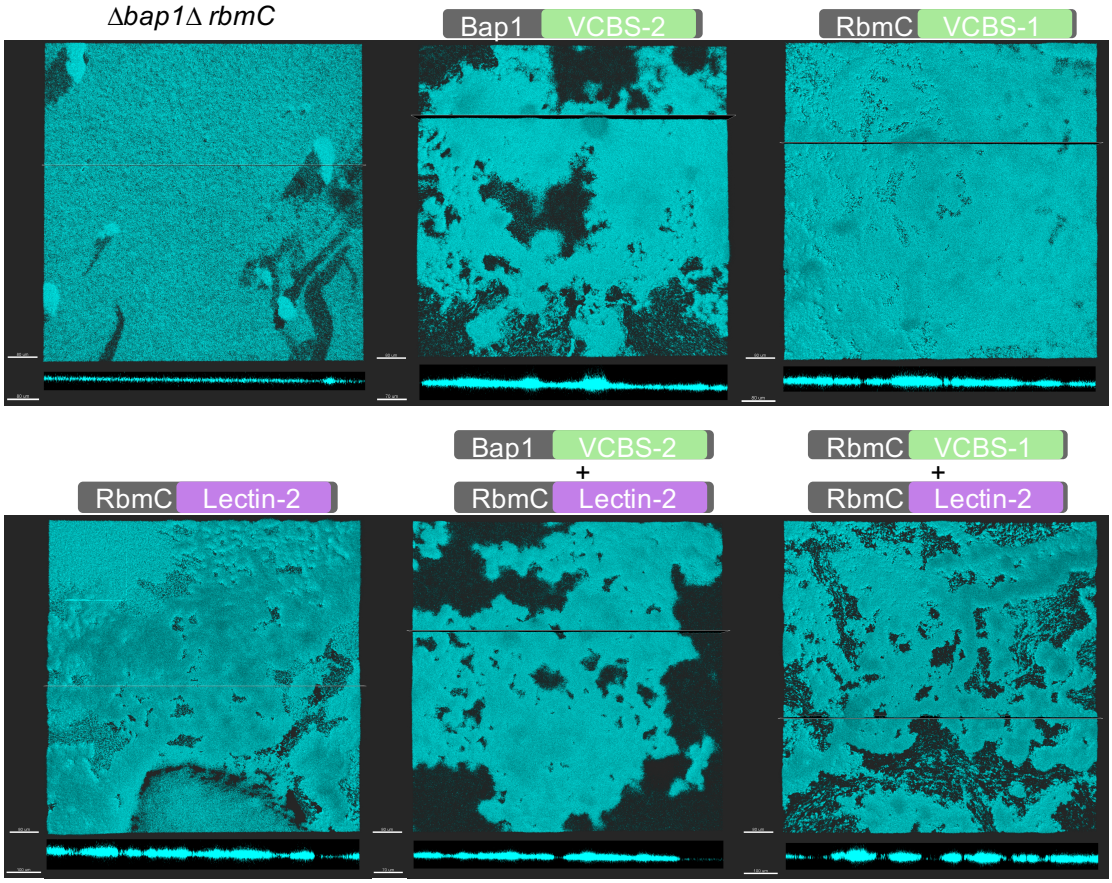

Δbap1Δ rbmC
Bap1 VCBS-2
RbmC VCBS-1
RbmC Lectin-2
Bap1 VCBS-2
+
RbmC Lectin-2
RbmC VCBS-1
+
RbmC Lectin-2

Figure 2.8. Impact of exogenous matrix protein domains on static biofilms lacking Bap1 and RbmC. Purification of Bap1 and RbmC domains (top) and their purity prior to addition to biofilms. Bap1-VCBS-2 (37 kDa) and RbmC-Lectin-2 (44 kDa) were purified and added containing a His-GST tag. RbmC-VCBS-1 (62 kDa) was purified, the tag removed (35 kDa) for addition. Exogenous addition of each of these respective proteins to a Δ*bap1*Δ*rbmC* is shown (bot) at 2.5 μM. Biofilms were grown for 8 hours, media removed, washed, and imaged using a 488 nm GFP channel.

Matrix protein interactions

Due to the enhancement of biofilm formation in Δ*bap1*Δ*rbmC* strain upon the endogenous and exogenous addition of the VCBS and Lectin domains, we next tested the possibility that VCBS and Lectin domains, or the full length RbmC and Bap1 protein could interact other matrix components such as the matrix protein RbmA and exopolysaccharide VPS. We used co-immunoprecipitation assay to analyze matrix protein interaction. Shown in *Figure 2.9* co-immunoprecipitation of Bap1 and RbmC when probing cell lysate and culture supernatant with α-Myc-RbmA indicates that both Bap1 and RbmC are able to interact with RbmA. Confirmation of these results is shown with co-immunoprecipitation of α-HA-Bap1 and α-FLAG-RbmC to probe for RbmA, where RbmA is present. These results define one shared contribution of Bap1 and RbmC to biofilm formation is through interaction with the third matrix protein RbmA.

Lectin domains are known to bind to glycan and both Bap1 and RbmC contain Lectin domains. Thus, we next tested for interaction of Bap1 and RbmC with the VPS polysaccharide. Shown in *Figure 2.10*, co-immunoprecipitation with VPS as bait, RbmA and Bap1 were found to interact with VPS however, RbmC is absent. The residues responsible for this interaction are currently unknown, and no glycan analysis has currently been conducted on Bap1.

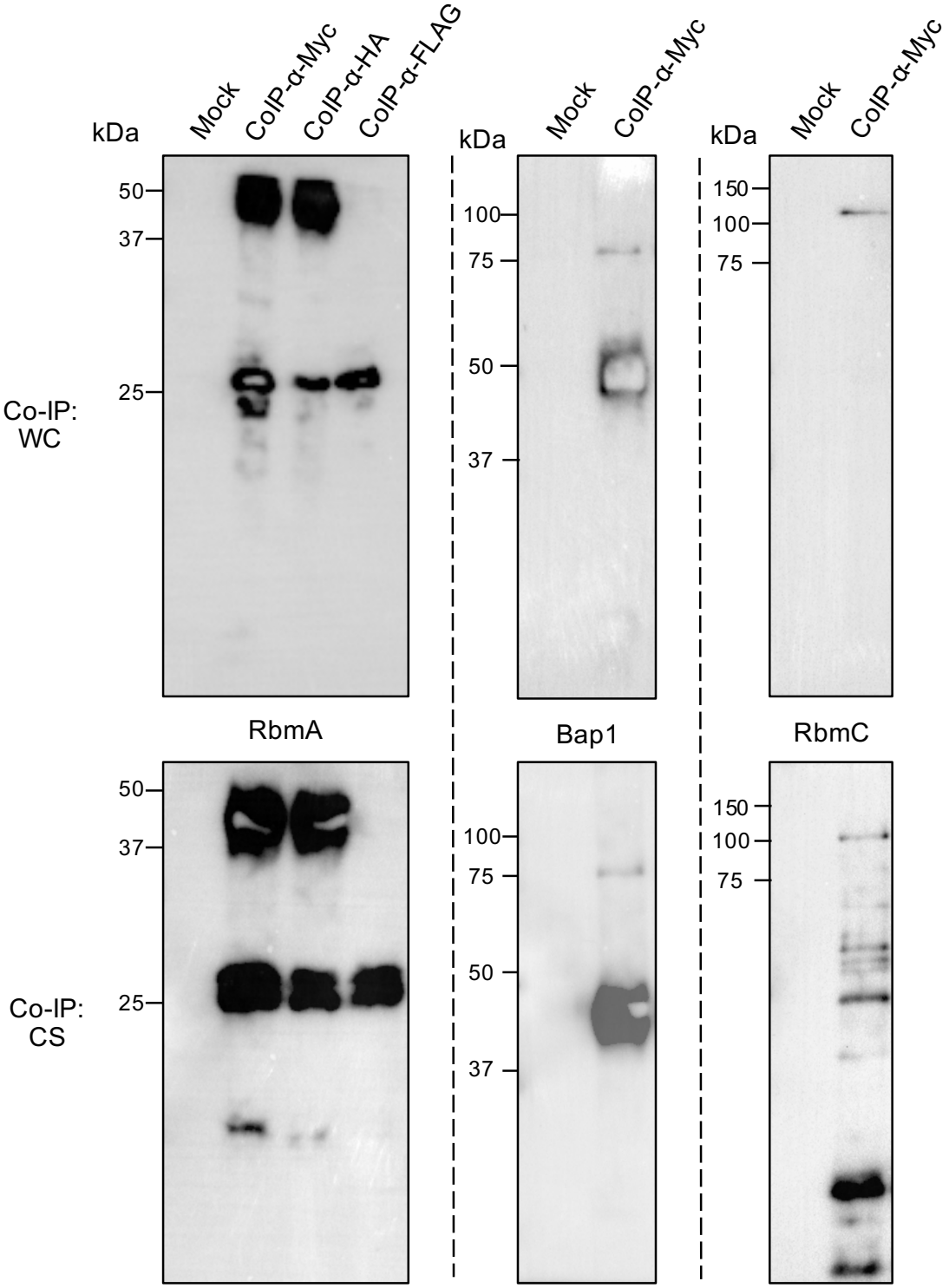

Mock
CoIP-α-Myc
CoIP-α-HA
CoIP-α-FLAG
kDa
50
37
25
Co-IP: WC
RbmA
Co-IP: CS
Mock
CoIP-α-Myc
kDa
100
75
50
37
Bap1
Mock
CoIP-α-Myc
kDa
150
100
75
RbmC

Figure 2.9. Interaction of matrix proteins with RbmA. Co-immunoprecipitation was conducted with whole cell lysate (WC) and culture supernatant (CS) and from a RbmA-Myc, Bap-HA, RbmC-FLAG strain as bait . Three separate reactions are shown above with their respective antibody.

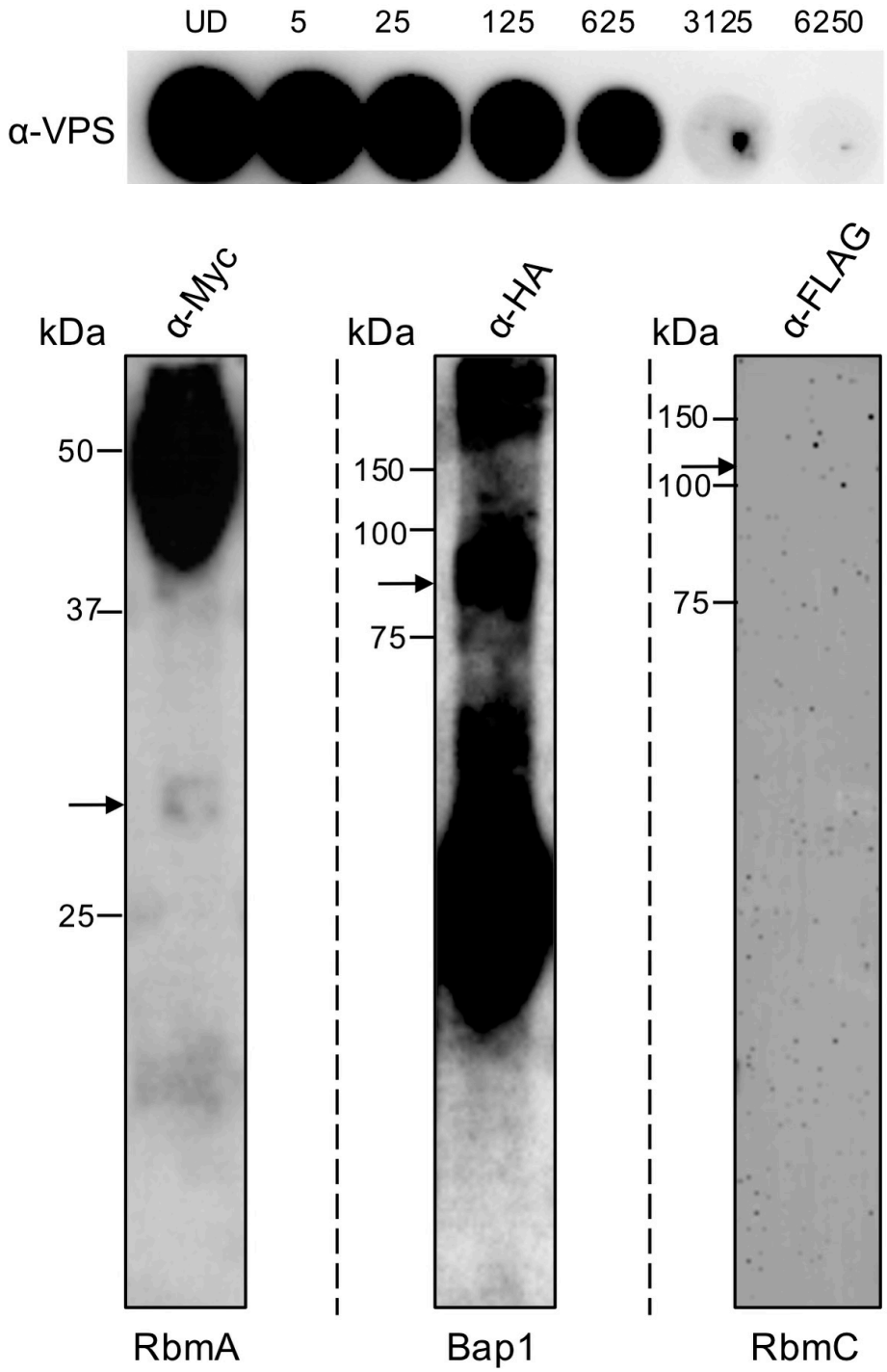
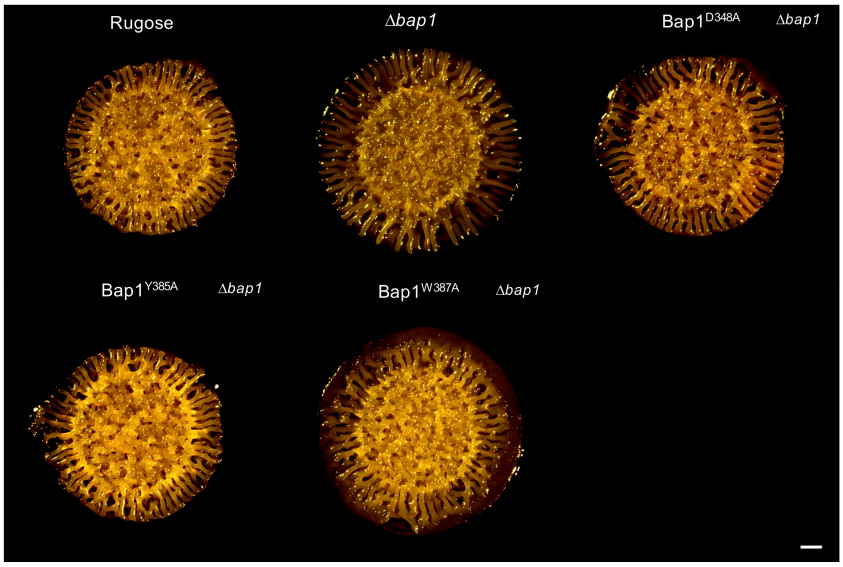

UD
5
25
125
625
3125
6250
α-VPS
α-Myc
α-HA
α-FLAG
kDa
kDa
kDa
50
37
25
150
100
75
150
100
75
RbmA
Bap1
RbmC

Figure 2.10. Interaction of matrix proteins with VPS is unique to RbmA and Bap1. Purification of VPS (top) from a Rugose parent strain shown using an α-VPS antibody that VPS is present. Co-immunoprecipitation (bottom) was conducted with VPS as bait and whole cell lysate from a RbmA-Myc, Bap-HA, RbmC-FLAG strain. Three separate reactions are shown above with their respective antibody.

Mutation of Bap1 and RbmC Lectin domains impacts biofilm architecture

In order to identify matrix protein residues that are required for biofilm architecture, we mutated individual residues within Bap1 and RbmC as shown in *Figure 2.11*. We identified residues that could be involved in glycan binding through bioinformatic analysis. We then replaced the wild type copy with mutated versions of RbmC and Bap1. We next analyzed changes to colony corrugation pattern as a read out of biofilm matrix protein and VPS interaction. Mutations show that Bap1's D348 and W387 may be involved in biofilm formation, although the point mutants are not as defective as total loss of *bap1*. Mutations in C-terminal residues of RbmC's second Lectin such as T870, N871, Y894, and W896 appear to impact biofilm formation to a greater extent than mutations in its first. These mutations result in a decrease in outer radial corrugation pattern seen in the Δ*bap1*Δ*rbmC* strain. These results suggest that Lectin domains play a role in the development of biofilm architecture.


Rugose
Δbap1
Bap1D348A Δbap1
Bap1Y385A Δbap1
Bap1W387A Δbap1

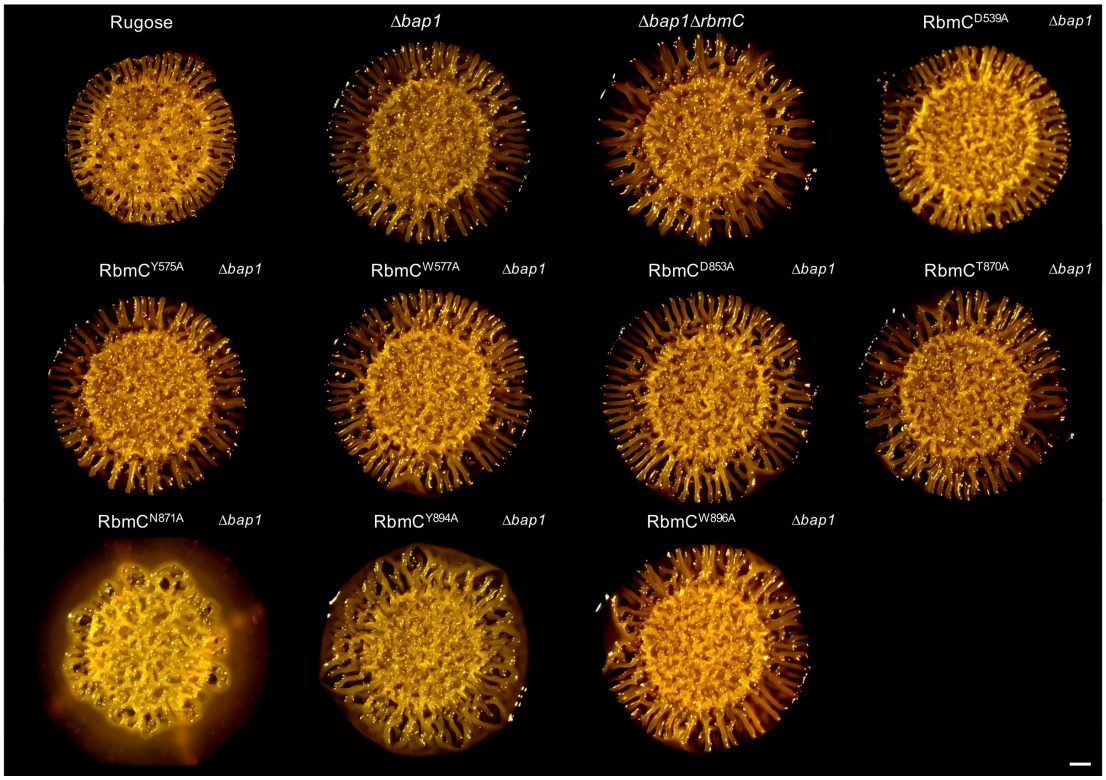

Rugose
Δbap1
Δbap1ΔrbmC
RbmCD539A Δbap1
RbmCY575A Δbap1
RbmCW577A Δbap1
RbmCD853A Δbap1
RbmCT870A Δbap1
RbmCN871A Δbap1
RbmCY894A Δbap1
RbmCW896A Δbap1

Figure 2.11. Bap1 and RbmC Lectin residue mutants impact biofilm formation. Shown above is the impact of Lectin residue point mutants on biofilm formation. Each spot consists of 3μL of *V. cholerae* overnight culture (1:200) plated in triplicate on 20 ml LB agar plates for 48 hr at 30°C followed by 24 hr at 25°C.

VPS and biofilm matrix proteins distinctly contribute to biofilm formation

Matrix proteins and VPS have been established as crucial for biofilm formation, however, the impact of their relative amounts has yet to be explored. To test the impacts of relative changes in matrix proteins and VPS, we grew overnight cultures of strains lacking all three matrix proteins or VPS and incubated them at different ratios. Shown in *Figure 2.12,* increasing production of matrix proteins increases the biofilm corrugation pattern. This increase however has a relative maximum, as the highest levels of matrix protein production lead to a decrease in corrugation supporting the importance of their relative abundance.

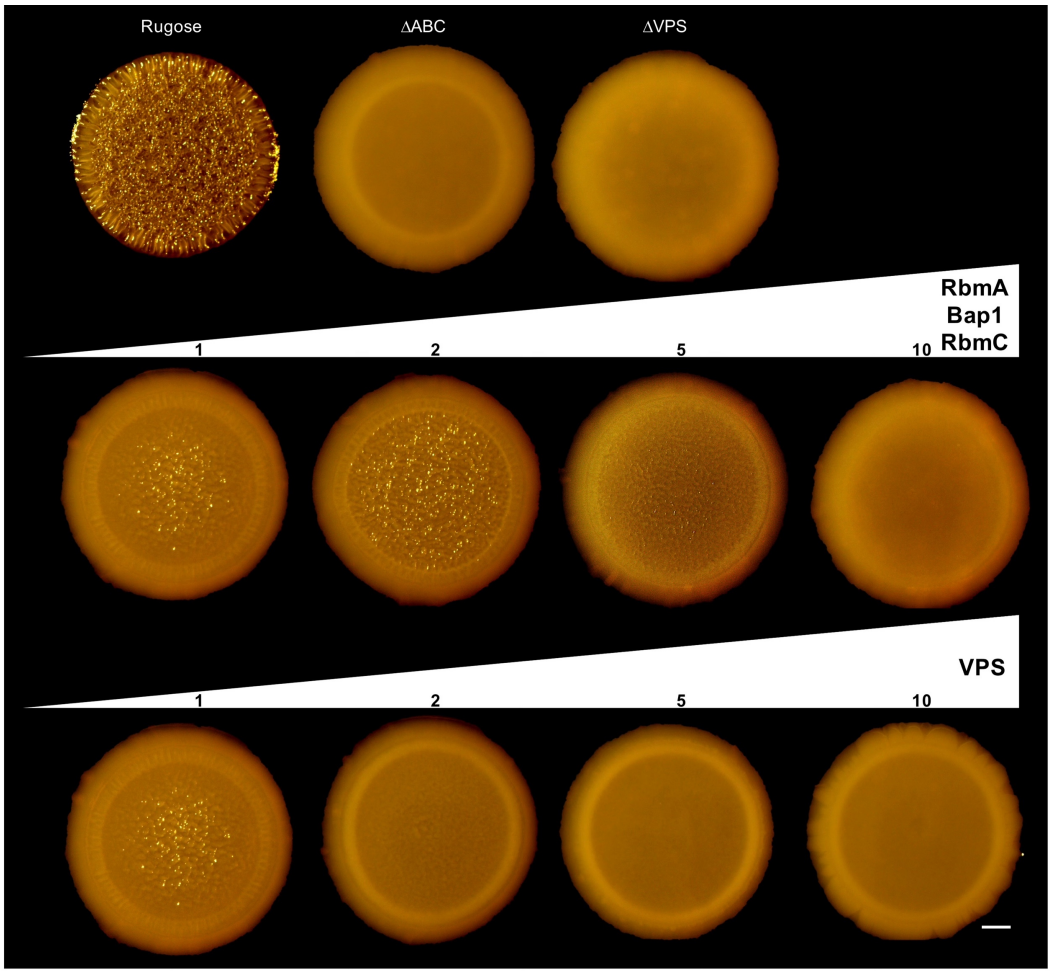

Rugose
ΔABC
ΔVPS
RbmA
Bap1
RbmC
1
2
5
10
VPS
1
2
5
10

Figure 2.12. Biofilm matrix proteins and VPS differentially contribute to biofilm formation. Shown above is the impact of alterations of biofilm matrix component ratios on biofilm formation. Each spot consists of 3µL of *V. cholerae* overnight culture plated in triplicate on 20 ml LB agar plates for 24 hr at 30°C

Bap1 and RbmC are primarily degraded by the HapA protease

Matrix protein degradation has been studied where RbmA is proteolyzed primarily by HapA along with PrtV and IvaP.[108] This finding allowed us to question whether HapA might be involved in the proteolysis of the other two matrix proteins Bap1 and RbmC. To test this possibility, we deleted *hapA* and probed for matrix protein stability over time. Shown in *Figure 2.13* is a western blot of spot biofilms after 24 hours of growth. It is important to note that under the conditions tested, in the parent strain, RbmC and Bap1 are not detected. However, in the strain lacking HapA, abundance of these proteins is markedly increased. These results suggest that HapA plays a major role in Bap1 and RbmC proteolysis.

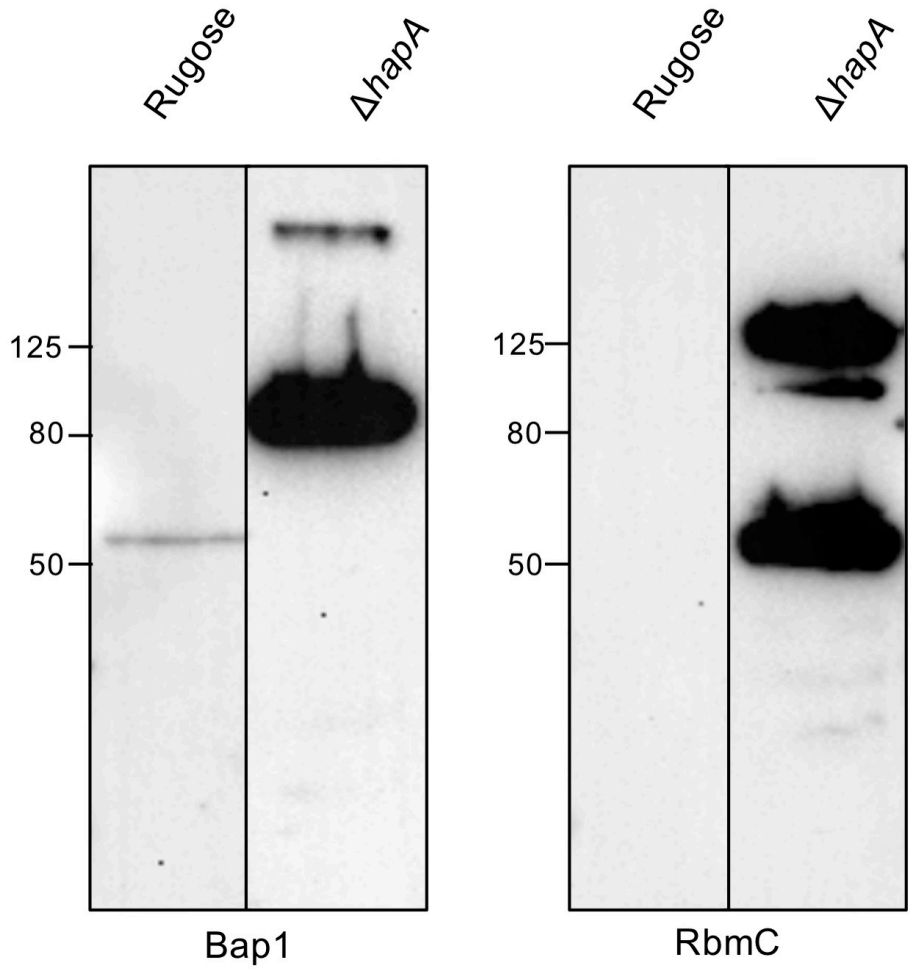

Rugose
ΔhapA
125
80
50
Bap1
Rugose
ΔhapA
125
80
50
RbmC

Figure 2.13. HapA is primarily responsible for Bap1 and RbmC proteolysis. Western blot probing for Bap1 and RbmC from WT and Δ*hapA* spot biofilms. Each spot was grown on a 20 ml LB agar plates for 24 hr at 30°C.

Discussion

In this study, we assessed the contribution of the biofilm matrix proteins Bap1 and RbmC and their individual domains to biofilm formation. We determined that each Bap1 and RbmC domain contributes to biofilm architecture through endogenous expression. This expression was sufficient for differences of cell-cell spacing observed upon the production of either of the three RbmC constructs through scanning electron microscopy. We determined that VCBS domains in both Bap1 and RbmC conferred an increase in surface adhesion under flow conditions. These results explain how single deletions do not result in a significant adhesion defect for flow conditions.[115] We next determined that addition of VCBS and Lectin domains from Bap1 and RbmC can increase biofilm formation in the Δ*bap1*Δ*rbmC* strain. We then determined that both Bap1 and RbmC interact with RbmA. Our initial studies using VPS Co-IP analysis, suggest that Bap1 could bind to VPS while RbmC cannot. However, the resolution of these studies are limited and additional VPS- matrix protein interaction studies need to be performed. We also determined that biofilm architecture is altered during mutation of conserved resides within Bap1 and RbmC. Mutation Bap1's D348 and W387 appear to change biofilm architecture greatest. Mutation of RbmC's second Lectin appear to impact biofilm architecture greater than mutations in its first. These results coincide with our previous results where removal of RbmC's second Lectin in the Δ*bap1*Δ*rbmC* background does not behave identically to a Δ*bap1* strain under any of the conditions we tested.

We additionally demonstrate that for optimum biofilm formation, relative ratios of the VPS and biofilm matrix protein components are important. We next show that HapA is the protease primarily responsible for Bap1 and RbmC proteolysis, in addition to its previously determined target RbmA.[28]

Perspectives

It is currently unknown what residues within matrix proteins are responsible for interaction with RbmA, as RbmA contains a RGD motif implicated in protein-protein interactions of fibronectin domains with integrin domains.[37,40] Though structural information is available on integrin-fibronectin interactions, the predicted fold of both Bap1 and RbmC appears to be a beta-propeller with little information regarding similar structures.[37–40] The interaction with VPS presents a mechanism by which matrix proteins differentially contribute to biofilm formation. This interaction may be conducted by the Lectin domain of Bap1, however, the VCBS annotated *Agrocybe aegerita* Lectin 2 has shown direct interaction with β1-4 branched GlcNAc glycans and its structure has been determined as a seven-blade β-propeller.[116] Overall, these results indicate that Bap1 and RbmC both contain VCBS and Lectin domains with distinct roles in biofilm formation. We then establish that Bap1 and RbmC interact with RbmA, and that Bap1 but not RbmC interacts with the major exopolysaccharide VPS.

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