Disruption of *Staphylococcus epidermidis* Biofilms by Compounds Induced by Dictyostelid *Polysphondylium pallidum*

By

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ABSTRACT

Biofilms, recognized as an accumulation of surface-attached cells surrounded by a matrix of one or more extracellular polymeric substances, account for more than 80% of human microbial infections, and it is commonly accepted that either most or all bacterial species are capable of forming biofilms. Due to the protective effect conferred by biofilm extracellular polymeric substances, biofilm-forming bacteria avoid immune recognition and clearance in chronic wounds and tissue- or device-related infections. Considering the biofilm phenotype's contribution to intractable infections and antibiotic tolerance, it is desirable to identify compounds capable of disrupting biofilms. Dictyostelids, soil dwelling bacterivores that inevitably encounter biofilms in their environment, have recently been observed to efficiently consume *in vitro* biofilms of multiple bacterial species. Given these observations, there is an opportunity to identify compounds potentially produced by Dictyostelid (*Polysphondylium pallidum*) consumption of *Staphylococcus epidermidis* biofilms that are responsible for biofilm disruption. As demonstrated here, this ability of biofilm disruption can be separated from live Dictyostelid cells, and candidate proteins responsible for this activity are identified.

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CHAPTER 1. INTRODUCTION

A public announcement released by the National Institutes of Health indicating that biofilms account for over 80% of microbial infections[1] highlights the need for solutions to address infections that are caused or exacerbated by biofilm formation. It has also been recognized that the majority of bacterial species, if not all, are capable of forming biofilms.[2,3] Biofilms allow bacteria to become hundreds- to thousands-fold more tolerant to antibiotics than their planktonic counterparts and represent a ubiquitous mechanism by which bacteria can withstand antibiotic treatment regimens.[4-6] Tissue related infections of biofilms cause osteomyelitis,[7] urinary tract infections,[8] dental plaque,[9] kidney stones,[10] endocarditis,[11] chronic tonsillitis,[12] chronic wounds,[13] and are leading cause of morbidity and mortality in cystic fibrosis.[6,14-16] In addition to tissue, these infections can occur on virtually all implanted devices in the human body such as pacemakers,[17] contact lenses,[18] vascular and urinary catheters,[19,20] breast implants,[21] and orthopedic implants.[15,16] The financial impact of chronic wounds alone costs the United States $25 billion annually,[13] and unofficial estimates place the annual financial burden for biofilm infections in the US at $94 billion and over 500,000 deaths.[22]

For *S. epidermidis*, biofilm formation is considered the primary virulence factor in the initiation and persistence of infection.[23] Biofilm-related infections of *S. epidermidis* are the most common pathogenic agent in capsular contraction of breast implants,[21] a host of implanted devices,[19] and cause 60% of cataract surgery-associated ocular infections.[24] It is also considered a leading cause of nosocomial sepsis and is the most frequently isolated organism from hospital-acquired infections.[25] Even within this specific segmentation, vascular catheter infections caused by *S. epidermidis* that progress to bloodstream infections are estimated to cost the US $2 billion annually.[26]

The use of thermotolerant Dictyostelids or their putative biofilm-disrupting secretions as anti-biofilm agents has been almost entirely unexplored.[27-29] Accordingly, previous demonstrations of Dictyostelids' ability to seemingly abrogate these bacterial biofilm defenses in multiple species of bacteria[27] have indicated potential for novel treatments targeting biofilm extracellular polymeric substances (EPS). The following investigation yields insights as to how Dictyostelids accomplish this feat and evaluate their therapeutic potential while establishing groundwork for future studies.

1.1 Biofilm Recalcitrance and Immune Evasion

Microbiological researchers during the previous century largely believed that bacteria only existed in disperse, natant forms, and this has been referenced to as 'the pure culture period'.[30] Accordingly, most research and development efforts concerning prospective antimicrobial drugs have traditionally been performed by treating bacteria grown in planktonic suspension. Although results derived from this type of investigation may be promising, this 'planktonic bias'[31] blinds these investigations to conditions caused by bacterial biofilm formation. Such conditions include the increase in antibiotic tolerance by multiple orders of magnitude,[4] the inhibition of immune recognition,[32] and the prevention of immune clearance.[33] Because biofilms represent the overwhelmingly predominant mode of existence of bacteria *in vivo*,[4] this makes statements such as "It is time to close the book on infectious diseases, and declare the war against pestilence won", often falsely attributed to United States Surgeon General Dr. William H. Stewart,[34] appear to be unqualified confidence.

The term biofilm refers to aggregated cells encased in a matrix of hydrated polymers optionally composed of polysaccharides, proteins, extracellular DNA (eDNA), lipids, and teichoic acids. This may refer to bacterial, fungal, or protist organisms and may also reference a combination thereof. Additionally, bacteria within a biofilm need not actually produce the polymer matrix of which they are surrounded themselves. The acknowledgement of biofilms in the literature will often be

accompanied by mention of association of cells with a surface, as this is the canonical first step in biofilm formation.[35] However, adherence to a surface is not necessarily required for pathogenic biofilm aggregates to form if cells have formed aggregates by adhering to themselves.[36] These 'suspended biofilms' still retain the hallmark increase in antibiotic tolerance,[36] and therefore, cellular aggregate formation should be considered the chief distinction of biofilm from monodisperse, planktonic cells.

It should be noted that antibiotic *tolerance* refers to the reversible reduction in susceptibility of bacteria to antibiotics, whereas antibiotic *resistance* is differentiated by what are considered permanent changes in this susceptibility such as mutations and plasmid-borne forms of genetic transfer. Although changes in antibiotic susceptibility with regard to biofilms are predominantly referred to as antibiotic tolerance because biofilms typically effuse susceptible, planktonic cells from their aggregates after their establishment, the observations that mutations and genetic transfers occur more often[37,38] and readily[39] within biofilms still warrant the use of the term resistance in conjunction with biofilms. Both terms represent an increase in the minimum inhibitory concentration (MIC) of an antibiotic which may result in the inability to eradicate pathogenic biofilms with an antibiotic regimen, termed 'recalcitrance'.

Previously, it had generally been thought that this antibiotic tolerance was primarily caused by biofilms preventing proper penetration of antimicrobial compounds.[40] Although there have been studies that have attempted to demonstrate that antibiotic penetration through biofilms has been responsible for this antibiotic tolerance,[41] slowing diffusion of nonspecific compounds cannot be the only cause as demonstrated by fluorescent tracer diffusion studies.[42] Thus, it is not surprising to find several examples in the literature where antibiotics have thoroughly penetrated biofilms without killing all biofilm-associated bacteria.[16] However, the biofilm EPS matrix does have

physiochemical properties that may slow the diffusion of select compounds such as binding to positively charged antibiotics[43] due to its ion-exchange capacity.[44]

Another characteristic of the biofilm lifestyle contributing to antibiotic tolerance is the physiological heterogeneity[45] of component cells. Nutrients and oxygen are depleted as they diffuse into the biofilm, creating gradients that force cells that are further within the biofilm into a slow-growing or dormant phenotype. These dormant cells are unaffected by antibiotics that require a growing phenotype, such as penicillin or cephalosporin, which act by inhibition of peptidoglycan synthesis[46] during cell replication, ultimately resulting in cell lysis and death. This form of antibiotic tolerance may be likened to that of so-called ‘persister cells’ which survive antibiotic-induced killing but do not acquire resistance as demonstrated by subsequent culture in antibiotic media with no change in the proportion of surviving cells.[47] Well after their first description in 1944 by Joseph Bigger,[47] persister cells have been recognized as one of the main mechanisms by which biofilm-forming bacteria develop antibiotic tolerance.[48,49] Cellular metabolism is also changed in certain biofilm subpopulations, not just in its overall rate, but the type of metabolic activity.[50] Bactericidal antibiotics whose activities depend on the generation of hydroxyl radicals[51] are rendered ineffective by the abandonment of aerobic metabolism which would otherwise generate fatal oxidative damage.[52]

The innate immune system has several signaling pathways capable of initiating responses to conserved bacterial motifs indicating the presence of a pathogen in host tissue. Common bacterial cell wall components such as lipopolysaccharide, lipoteichoic acid, and peptidoglycan are well-known ligands for receptors that initiate these responses. These pattern recognition receptors (PRRs) are also capable of recognizing biofilm EPS components. The Toll-like receptor (TLR) and NOD-like receptors are PRRs that can be stimulated by EPS components such as polysaccharide intercellular adhesin (PIA), eDNA, peptidoglycan, lipoteichoic acids, and phenol-

soluble modulin (PSM).[53,54] While *in vitro* experiments with purified PIA and PSM extracts from staphylococcal bacteria demonstrate clear TLR2 activation,[55,56] *in vivo* staphylococcal biofilms are able to prevent TLR2 activation despite the presence of TLR2 ligands.[53] Similar results have been shown for TLR9[53] and suggest that these defense mechanisms are successfully abrogated by the biofilm phenotype. Because neutrophils, macrophages, and dendritic cells express TLR2 and TLR9 and use them to recognize bacteria,[53,57] the innate immune response fails to correctly address biofilm infections. The mechanism by which TLR activation is prevented is yet unknown, but it has been suggested that it may be due to an unmet requirement of phagocytosis for proper recognition by macrophages.[58] The impact of biofilm on the immune system is also investigated by non-molecular measurement of innate immune responses, one of which is macrophage phagocytosis. For example, *in vitro S. epidermidis* biofilms display less susceptibility to phagocytosis than planktonic controls.[59] Even conditioned supernatants from *Staphylococcus aureus* biofilms have been shown to inactivate macrophage's phagocytosis of fluorescent beads or planktonic bacteria compared to naïve controls.[57]

As biofilm infections embody a substantial reservoir of antigens available for generation of adaptive immune responses, it can be expected that antibody-mediated reactions are hindered or subverted by the biofilm phenotype.[60] Although antibodies appear to have no problem penetrating biofilms,[61] antibody-assisted opsonization can still be side-stepped, as with innate immune responses, by prevention[62] or inactivation of phagocytosis.[57] Prophylactic immunization with *S. aureus* biofilm exoproteins, but not planktonic exoproteins, causes significant reduction in bacterial cell numbers in *S. aureus* biofilm infections, while unassisted antibody generation from *de novo* responses to biofilm infections was not as effective.[63] This supports the possibility that biofilms perform some strategy to prevent proper antibody generation. The co-opting of host extracellular matrix or serum proteins has been recognized as a common virulence factor for the purposes of attachment to host tissues;[64] however, others have described this activity may

camouflage antigenic sites on pathogenic bacteria.[65] Bacterial binding of host extracellular matrix proteins such as fibronectin,[64,66] fibrinogen,[23,67] or serum proteins such as albumin[65] may mask antigens and reduce the surface area available for opsonization and complement deposition.[68] Notably, *S. aureus* USA300 isolate LAC has also been shown to require the presence of such proteins that bind host extracellular matrix proteins to form biofilms.[64]

1.2 *S. epidermidis* Biofilm

S. epidermidis presents itself as a fitting model organism to study the impact of the biofilm phenotype on pathogenesis because biofilm formation is the primary virulence factor that distinguishes it from its otherwise commensal existence.[23,32] Its usual role on human skin as a commensal organism provides a pervasive reservoir for inoculation of susceptible tissues[7-13,69] and implanted medical devices.[15-21,70] Multispecies biofilms are often used as more relevant models of chronic wounds,[71] particularly for common pathogenic infections involving *S. aureus* and *P. aeruginosa*.[72] However, single-species biofilms of *S. epidermidis* are reported as the only causative agent for many tissue- and device-related biofilm infections.[69,70,73-75] Therefore, models employing single-species biofilms of *S. epidermidis* and treatments developed for these infections are still warranted despite intense focus on polymicrobial biofilms.

S. epidermidis biofilm EPS displays all three common matrix components: protein, polysaccharides, and extracellular DNA in addition to teichoic acids.[23,76] Its proteinaceous matrix components consist of Accumulation-associated protein (Aap), Extracellular matrix binding protein (Embp), and Bap homologue protein (Bhp) named after the *S. aureus* Biofilm-associated protein (Bap) of similar function.[23,77,78]

Aap is responsible for the formation of fibrillary tufts that extend just beyond the cell, contributing to the cell's surface hydrophobicity. The presence of Aap in *S. epidermidis* is exceedingly

common, as 90% of *S. epidermidis* strains harbor it.[79] In a rat catheter infection model, Aap was shown to be required for infection and initiate attachment to the catheter surface. Schaeffer *et al.* also identified the region conferring this activity as the Aap A domain through the use of isogenic mutants with or without the Aap A domain in addition to granting this adhesion capability to an adhesion-negative strain by use of a plasmid containing the A domain linked to a cell wall anchor. They offered a model whereby full-length Aap (including the exterior A domains and interior B domains) initiates attachment followed by protease cleavage of non-adhered A domains, allowing for truncated-Aap polymerization and cellular accumulation through intercellular B domain interactions.[80]

Embp has been primarily described as a fibronectin-binding protein. Of note is its full size, which is 1,100kDa and 460kDa for a truncated form.[23] The found-in-various-architecture (FIVAR) domains of Embp mediate the affinity to fibronectin, a host extracellular matrix protein, which allow for initiation of attachment to host tissues by which it is thought to mediate pathogenicity.[62] In addition to its attachment function via fibronectin binding, Embp has been shown as sufficient for *S. epidermidis* biofilm formation in strains lacking both the PIA and Aap canonically associated with *S. epidermidis* biofilm formation.[23,62] Christner *et al.* noted that, curiously, previous studies had indicated that PIA-negative strains found negative for *in vitro* biofilm formation were equally pathogenic in a guinea pig infection model.[62,81,82] Considering that standard *in vitro* culture of *S. epidermidis* only elicits low expression for Embp;[62] and the inclusion of serum in media, and therefore fibronectin, induces high levels of *S. epidermidis* Embp expression and cellular aggregation, perhaps these prior studies were witnessing Embp's involvement only in *in vivo* but not *in vitro* conditions. Embp's biofilm formation ability also potentially contributes to virulence by inhibition of *in vitro* phagocytosis by macrophages, extending its pathogenic function beyond that of just attachment.[62]

Identification of *S. epidermidis* Bhp, encoded by the *S. epidermidis bap* gene, as a homologue of Bap studied in *S. aureus* was based on a high sequence similarity of Bap to Bhp found in that of a strong biofilm-forming strain of *S. epidermidis* RP62A.[77] Bhp displays similar cell wall anchor, membrane spanning, and cell surface protein domains as seen in Bap.[77] Further investigation confirmed its biofilm-forming capabilities by heterologous complementation of the *S. epidermidis bap* gene in PIA-negative, biofilm-deficient *S. aureus* that conferred biofilm formation.[77] Accordingly, disruption of the *S. epidermidis bap* gene abrogated biofilm formation.[77]

Teichoic acids (TA), comprised of repeating sugar and phosphate moieties, are considered to be universal surface components of Gram-positive bacteria[78] and optionally present as extracellular matrix components of biofilms.[83] Their discovery as extracellular components in *S. epidermidis* were first reported by Hussain *et al.* and confirmed the similarity in composition to TA associated with the cell wall.[83,84] Similar to the function of Embp, *S. epidermidis* TA significantly enhances fibronectin binding that can assist in attachment to host tissues.[69,83]

The exopolysaccharide *S. epidermidis* biofilm component PIA is a homopolymer chain of β-1,6-linked *N*-acetylglucosamine.[76] This positively charged and partially deacetylated chemistry is also referred to as poly-β(1-6)-N-acetylglucosamine (PNAG).[85] Synthesis of PIA is controlled by the *icaADBC* locus, first discovered by Heilmann *et al*.[85,86] Because PIA-positive *S. epidermidis* strains have been often isolated from infections of medical implants and few commensal *S. epidermidis* isolates possess *icaADBC*, it is not surprising to find that carriage of *icaADBC* by *S. epidermidis* comes at the cost of fitness on human skin.[23,87] Therefore, PIA is considered an important virulence determinant for *S. epidermidis*. Aarag *et al.* demonstrated that a *S. epidermidis* PIA-negative mutant generates significantly increased cytokine response to biofilm *in vitro* compared its isogenic and PIA-positive control, suggesting that biofilm-associated PIA is potentially responsible for attenuating innate immune responses.[88] They also demonstrated that

these differences are diminished when these strains are grown as a planktonic suspension culture.[88] Purified PIA has also been identified as potent complement activator.[88] It has been noted[76] that, although strong complement activation prevails in biofilm infection of *S. epidermidis*, C3b and IgG deposition on the biofilm was markedly decreased compared to planktonic controls.[89] Cerca *et al.* applied opsonic antibodies targeting *S. epidermidis* PNAG to both planktonic and biofilm conditions and observed that, even though biofilm cells produced more PNAG per cell, biofilm displayed reduced opsonophagocytic removal. These opsonic antibodies were also determined to completely penetrate the biofilm, indicating that the biofilm matrix environment did not prevent non-specific diffusion.[61] These observations display adherence of *S. epidermidis* biofilms to a common theme in biofilm infections that the environment of the biofilm matrix impedes the response of the immune system.

1.3 Dictyostelids and Their Predation on Bacteria

Dictyostelids are heterotrophic, social amoebae that consume bacteria in soil via phagocytosis. As single myxamoebae, they undergo mitotic division as they feed on bacteria until their prey populations are exhausted, upon which time they aggregate and progress through several cooperative, multicellular stages that culminate in the production of dormant spores perched above stalk structures. In some cases, Dictyostelids can also ingest surrounding molecules via macropinocytosis without the need for the presence of bacteria; this is termed ‘axenic growth’. Loss of the gene encoding RasGAP Neurofibromin (NF1) allows Dictyostelids to grow axenically via the increase of macropinocytosis capacity.[90]

Because the Dictyostelid feeding process naturally occurs within the soil environment where the majority of bacteria are expected to exist as biofilms,[91] Dictyostelids inevitably encounter this defense of bacteria. Owing to the aegis of natural selection, they would be expected to have developed mechanisms to circumvent this phenomenon to phagocytose their prey. Sanders *et al.*

have recently described the ability of several Dictyostelids to disrupt biofilms during bacterial predation; however, the mechanism by which they do so has yet to be described.[27]

1.4 Current Biofilm Disruption and Prevention Strategies

Many bacterial biofilm solutions are centered around the prevention of biofilm formation such as inhibition of quorum sensing that initiates the production of EPS components.[92] Because these treatments are often only successful in preventing formation of biofilms but not the disruption of already-formed biofilms, their efficacy is limited to less-relevant prophylactic use.[93] Due to this drawback, there exists a significant need for treatments able to remove already-formed biofilms present in biofilm-related infections. Previously, it has been shown that mature biofilms are susceptible to harsh, nonspecific enzymatic activity by proteases such as pronase and proteinase K.[94] However, non-specific proteases are not useful in a medical setting as they would similarly harm patient tissues. If the activity involved in the degradation of biofilm EPS by Dictyostelids is specific to the chemistries of the biofilm EPS itself, it may serve as a safe and effective treatment for biofilm-related infections.

Anti-biofilm compounds that are capable of disrupting already-formed biofilms have previously been discovered such as peptides mimicking those from the human immune system with broad-spectrum anti-biofilm activity.[95] However, few anti-biofilm drugs have been commercialized,[96,97] and, given the broad range of species' biofilms that Dictyostelids can successfully disrupt, broad-spectrum anti-biofilm drugs could presumably be discovered by isolation of active compounds from Dictyostelid secretions. The discovery of nonantibiotic compounds with broad-spectrum anti-biofilm activity for the eradication of biofilm infections have been referred to as modern 'holy grails'.[94] Their potential use to re-sensitize biofilm-encased bacteria to antibiotics displays an exciting new class of drugs to treat intractable infections.

The work portrayed in this thesis investigates the hypothesis that Dictyostelids, specifically *Polysphondylium pallidum*, produce compounds that aid in their predation of biofilm encased bacteria. The following chapters address whether this observed anti-biofilm activity can be separated from live *P. pallidum* amoebic cells, the identity of the active compound, and potential adjuvant uses of the compound against biofilm-forming *S. epidermidis*.

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CHAPTER 2. SOLUBLE MACROMOLECULAR COMPOUNDS INDUCED BY *P. pallidum* PREDATION DISPERSE *S. epidermidis* BIOFILM EPS

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2.1 Abstract

Dictyostelids have recently been shown to harbor the ability to disrupt bacterial prey biofilms. If this activity can be separated from live Dictyostelid cells, then candidate proteins could be identified for potential therapeutic applications against bacteria that are tolerant to antibiotics. Electron microscopy as well as macroscopic staining of biofilms was used to show that biofilm EPS of *S. epidermidis* is removed by compounds yielded from feeding *S. epidermidis* to Dictyostelid *P. pallidum*. Size fractionation of these compounds indicated that either macromolecules or small molecules in conjunction with macromolecules are responsible for this anti-biofilm activity.

2.2 Introduction

Due to the protective effect conferred by biofilm EPS, biofilm-forming bacteria avoid immune recognition and clearance in chronic wounds[1,2] and tissue- or device-related infections.[3,4] Bacterial biofilm formation is typically recognized as an accumulation of surface-attached cells surrounded by a matrix of one or more extracellular polymeric substances usually consisting of DNA, protein, and polysaccharides. A portion of cells encased within this EPS become metabolically dormant, circumventing lethal activity of antibiotics that require metabolic activity or growth. Therefore, eliminating biofilm EPS may allow previously tolerant bacteria to become susceptible to antibiotics. Many bacterial biofilm solutions are centered around the prevention of biofilm formation such as inhibition of quorum sensing that initiates the production of EPS components.[5] Because these treatments are often only successful in preventing formation of

biofilms but not the disruption of already-formed biofilms, their efficacy is limited to less-relevant prophylactic use.[6] Due to this drawback, there exists a significant need for treatments able to remove already-formed biofilms present in biofilm-related infections.

Staphylococcus epidermidis is an appropriate model organism to investigate the impact of biofilm formation on pathogenesis because the biofilm-forming phenotype is the primary virulence factor that distinguishes it from its commensal existence.[1,7] *S. epidermidis* infections associated with biofilm formation constitute a majority of hospital-acquired sepsis cases, and *S. epidermidis* is the most frequently isolated organism from nosocomial infections.[8] Therefore, models employing single-species biofilms of *S. epidermidis* and treatments developed for these infections are still warranted despite intense focus in the literature on polymicrobial biofilms.

It has recently been demonstrated that Dictyostelids, free-living phagocytic amoebae that act as bacterial predators in soil, have the ability to feed on biofilm-forming bacteria despite their protective matrix.[9] Because the majority of bacteria are expected to exist as biofilms in all but the most copiotrophic environments,[10-12] it would be no surprise that they exploited evolutionary incentives to access their prey by production of—or inducing bacterial prey to generate—compounds that disperse biofilm EPS. The use of Dictyostelids or their putative biofilm-disrupting products as anti-biofilm agents has been almost entirely unexplored.[9,13,14] Accordingly, previous demonstrations of Dictyostelids' ability to seemingly abrogate these bacterial biofilm defenses in multiple species of bacteria[9] have indicated potential for novel treatments targeting biofilm EPS. The present chapter investigates the anti-biofilm activity of compounds whose production is induced by *Polysphondylium pallidum* El Salvador feeding on *S. epidermidis* referred to as Dictyostelid-induced anti-biofilm compounds (D-IABC).

2.3 Methods

2.3.1 Strains used for experiments

To evaluate the potential specificity of degradation for certain biofilm EPS components, one strain of *S. epidermidis* and its biofilm-deficient variant were used to form biofilms. The *S. epidermidis* used is a strong biofilm-forming strain originally isolated from an infected central venous catheter (*S. epidermidis* 1457) referred to as AH2490.[15,16] Next is its attenuated biofilm former variant *S. epidermidis* 1457 Δica::dhfr, an isogenic insertional mutant of AH2490 containing dihydrofolate reductase inserted into the *ica* operon responsible for PIA synthesis, referred to as AH2589.[16,17] All experiments employ AH2490 to produce biofilms and D-IABC unless otherwise noted. *P. pallidum* El Salvador is a thermotolerant Dictyostelid strain capable of growth at 37°C isolated from San Salvador, El Salvador.[18]

2.3.2 S. epidermidis Biofilm Formation

S. epidermidis was grown in 3% (w/v) tryptic soy broth (Fisher Scientific, Pittsburgh, United States, Catalog # DF0370-17-3) overnight at 37°C and 30 µL of resulting culture was applied to sterile Isopore 0.2 µm polycarbonate membranes (EMD Millipore, Burlington, Massachusetts, United States, Catalog # GTTP02500) above tryptic soy agar. Inoculated membranes were allowed to absorb culture briefly and were then incubated for 48 hours at 37°C to form biofilms on the membrane surface.

2.3.3 Generation and Application of P. pallidum D-IABC

Once mature *S. epidermidis* biofilms were formed, the polycarbonate membrane-bound biofilms were aseptically transferred to 5 mM potassium phosphate-buffered agar within a 6-well plate (Corning, New York, United States, Catalog # 353046) and $2x10^4$ *P. pallidum* spores were applied to begin predation and generate Dictyostelid-induced compounds for 96 hours at 28°C. After

feeding was complete and sporangia were evident on the membrane surface, the membranes were gently transferred and placed onto fresh buffer agar with the sporangia-containing side of the membrane facing the agar. Atop the still-sterile face of this now D-IABC-laden membrane, another *S. epidermidis* biofilm membrane was placed with its biofilm facing upward to receive secreted materials through the membrane during overnight incubation at 28°C (Figure 2.1). In experiments where only D-IABC solution was used to treat biofilms on polycarbonate membranes, 30 µL of D-IABC was gently pipetted onto the biofilm before overnight incubation at 37°C.

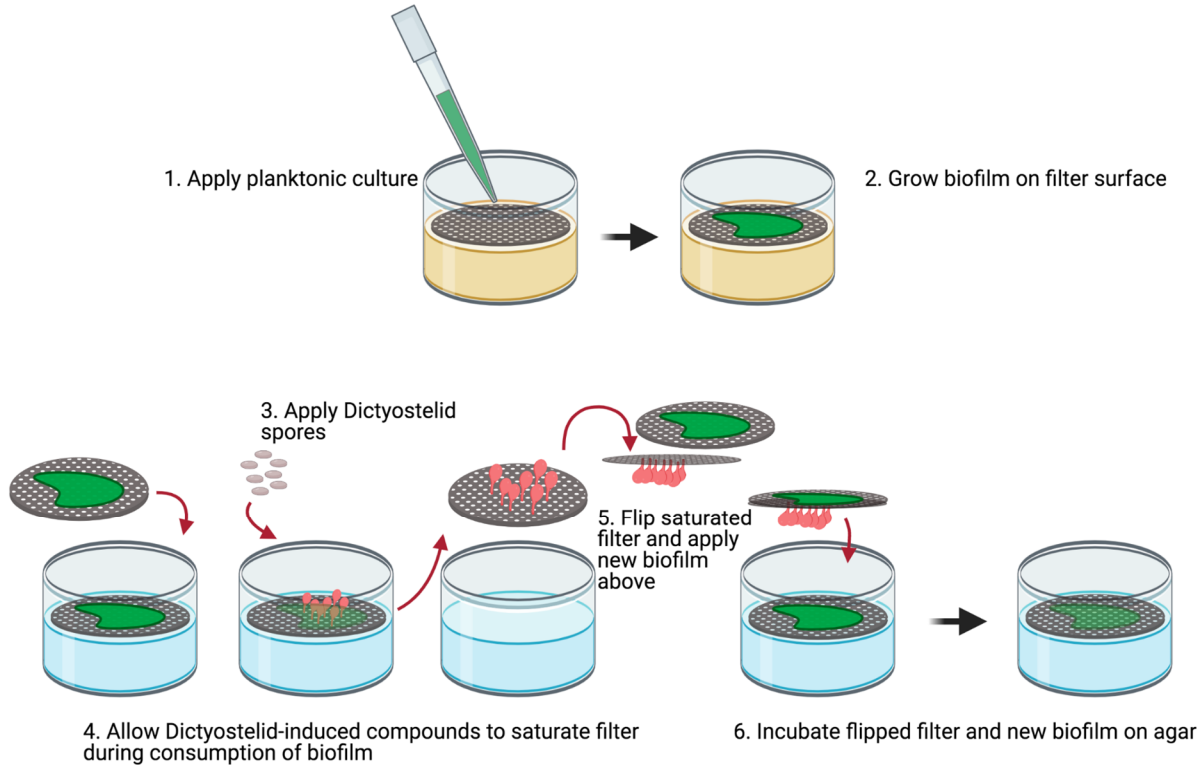

Figure 2.1 Establishment of S. epidermidis biofilms and generation of D-IABC. Planktonic *S. epidermidis* cultures were applied to sterile polycarbonate membranes above tryptic soy agar to initiate biofilm formation. Polycarbonate membrane-bound *S. epidermidis* biofilms were transferred to potassium phosphate-buffered agar and *P. pallidum* spores were applied to generate D-IABC within the membrane. Following *P. pallidum* spore treatment, membranes laden with D-IABC were transferred to fresh buffer agar. Newly prepared *S. epidermidis* biofilms were positioned above polycarbonate membranes containing *P. pallidum* D-IABC to induce biofilm disruption. Negative controls employed *S. epidermidis* biofilms without the addition of Dictyostelid spores.

Unfractionated D-IABC produced in suitable quantities for chromatographic purification experiments in this work was generated by feeding heat-killed *S. epidermidis* AH2490 to *P. pallidum* on a filter cake of diatomaceous earth. Briefly, 500 mL of overnight *S. epidermidis* culture was autoclaved at 121°C and 103.4 kPa for 45 minutes before vacuum application to a filter cake of 15 g of Celpure C-1000 diatomaceous earth (Imerys, Paris, France) above a grade 454 filter paper (VWR, Radnor, Pennsylvania, United States, Catalog # 28306-095) in a 9 cm diameter Büchner funnel. This filter cake of diatomaceous earth was previously established by vacuum application to the filter paper while suspended in normal saline. After application of overnight culture and subsequent rinsing with 20 mM potassium phosphate pH 6.5 via vacuum, *P. pallidum* spores were gently pipetted onto the surface bacteria. This arrangement was carefully transferred out of the Büchner funnel and into a 100 mm plate (Fisher Scientific, Pittsburgh, United States, Catalog # 08-757-100D) before incubation at 28°C for 96 hours. After incubation and subsequent suspension in 10 mM sodium phosphate (VWR, Radnor, Pennsylvania, United States, Catalog # JT3827-1) at pH 6.5, D-IABC solution was recovered by vacuum filtration (Sartorius, Göttingen, Germany, Catalog # 14555994).

2.3.4 Washing and Staining Remaining S. epidermidis on Polycarbonate Membranes

Control and D-IABC-exposed *S. epidermidis* biofilm membranes were transferred to a 15 mL conical tube (Thermo Scientific, Waltham, Massachusetts, United States, Catalog # 339651) and 2 mL of deionized water was added and used to rinse away cells not bound to the membrane surface by 5 gentle inversions for each membrane. Membranes were then transferred to agar plates containing 0.1% (w/v) Congo Red dye (Sigma-Aldrich, St. Louis, United States, Catalog # C6277) and incubated overnight at 4°C.

2.3.5 Size Fractionation of D-IABC

D-IABC was derived from agar beneath a polycarbonate filter membrane with 0.2 µm pores on which *P. pallidum* and *S. epidermidis* biofilms were incubated for 96 hours at 28°C. Liquid was extracted from the agar by a freeze-thaw cycle at -20°C and 37°C and centrifugation at 4,000 relative centrifugal force (RCF) for 5 minutes. This material was applied to fresh *S. epidermidis* biofilms for evaluation of anti-biofilm activity after sterile 0.22 µm syringe filtration (Fisher Scientific, Pittsburgh, United States, Catalog # 09-720-004) and subsequent filtration using an Amicon 3,000 nominal molecular weight limit filter (EMD Millipore, Burlington, Massachusetts, United States, Catalog # UFC900308) as directed by the manufacturer (Figure 2.2). Both the filtrate and reservoir retentate material was kept for activity assays. Following overnight incubation of treatment solutions at 28°C, biofilms underwent a gentle wash procedure as described above to remove non-adherent cells.

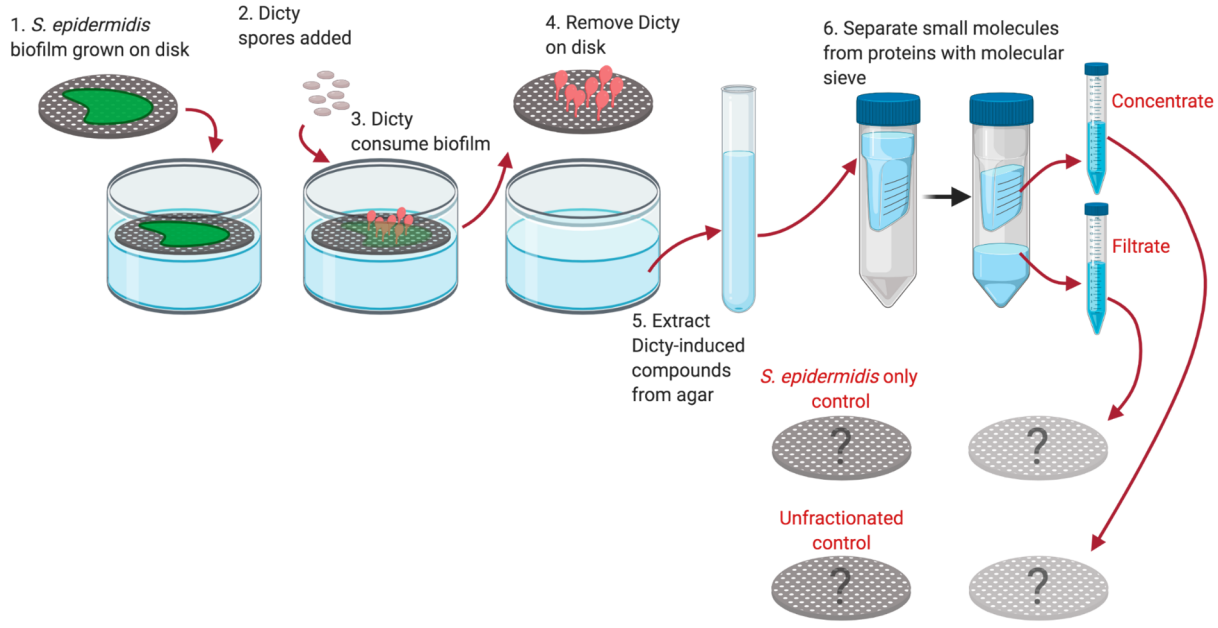

Figure 2.2 Diagram of *P. pallidum* D-IABC size fractionation procedure. D-IABC was allowed to diffuse into agar by feeding *S. epidermidis* to *P. pallidum* at 28°C for 96 hours while separated from the agar beneath by a polycarbonate membrane with 0.2 µm pores. Liquid extracted from the agar was then subjected to size fractionation using a 3,000 nominal molecular weight limit filter to separate small molecules from proteins and other macromolecules. *S. epidermidis*

AH2490 biofilms were treated with filtrate and retentate material to determine if small molecules were capable of anti-biofilm activity as seen in unfractionated D-IABC.

2.3.6 Scanning Electron Microscopy

S. epidermidis biofilms were fixed with phosphate-buffered 10% formalin (VWR, Radnor, Pennsylvania, United States, Catalog # MKH12109) for 1 hour. Sample solvent environment was switched to ethanol (Deacon Labs, Pennsylvania, United States, Catalog # 2701) with 50%, 70%, 80%, 90%, 100%, and 100% gentle ethanol incubations (10 minutes each) followed by Megasamdri critical point drying (Tousimi, Maryland, United States) and sputter coating with 5 nm of iridium with a Leica EM ACE 600 Sputter Coater (Leica Microsystems, Wetzlar, Germany). Scanning electron microscopy (SEM) was performed by a Hitachi S3400N scanning electron microscope (Hitachi, Tokyo, Japan).

2.3.7 Microplate Assay of Anti-Biofilm Activity

Similar to biofilm biomass measurement methods found in the literature,[19] a crystal violet stain recovery assay was used to determine remaining biofilm biomass after treatment. Briefly, overnight *S. epidermidis* culture (AH2490) diluted in fresh tryptic soy broth (at a dilution suitable to achieve a 0.5 McFarland Standard absorbance at 625 nm of bacteria suspended in normal saline) was used to inoculate tissue-culture treated polystyrene 96-well plates (Corning, New York, United States, Catalog # 353072) and initiate biofilm formation overnight at 37°C with 110 rpm orbital shaking. Subsequently, media was removed from biofilm wells and treatment solutions were added for overnight incubation at 37°C with 110 rpm orbital shaking. Following incubation, wells were rinsed with deionized water to remove unbound cells and remaining cells were stained with 0.1% (w/v) crystal violet (Sigma-Aldrich, St. Louis, United States, Catalog # C0775). After unbound crystal violet was removed with 3 subsequent rinses with deionized water, 30% (v/v) acetic acid (VWR, Radnor, Pennsylvania, United States, Catalog # 9526-03) was used to recover

stain. Absorbance of recovered crystal violet stain was measured at 590 nm with a Cytation 5 multi-mode reader (Biotek, Winooski, Vermont, United States).

Earlier experiments performed by our group employed a legacy version of the microplate assay in which unbound bacteria were not removed via a wash step before staining with crystal violet. This form of the assay allows for substantially less removal of biofilm biomass than if a wash prior to staining is performed. A direct comparison of these methods is shown in Figure 2.3. Figure legends will indicate whether this legacy assay method was used to produce data in this work.

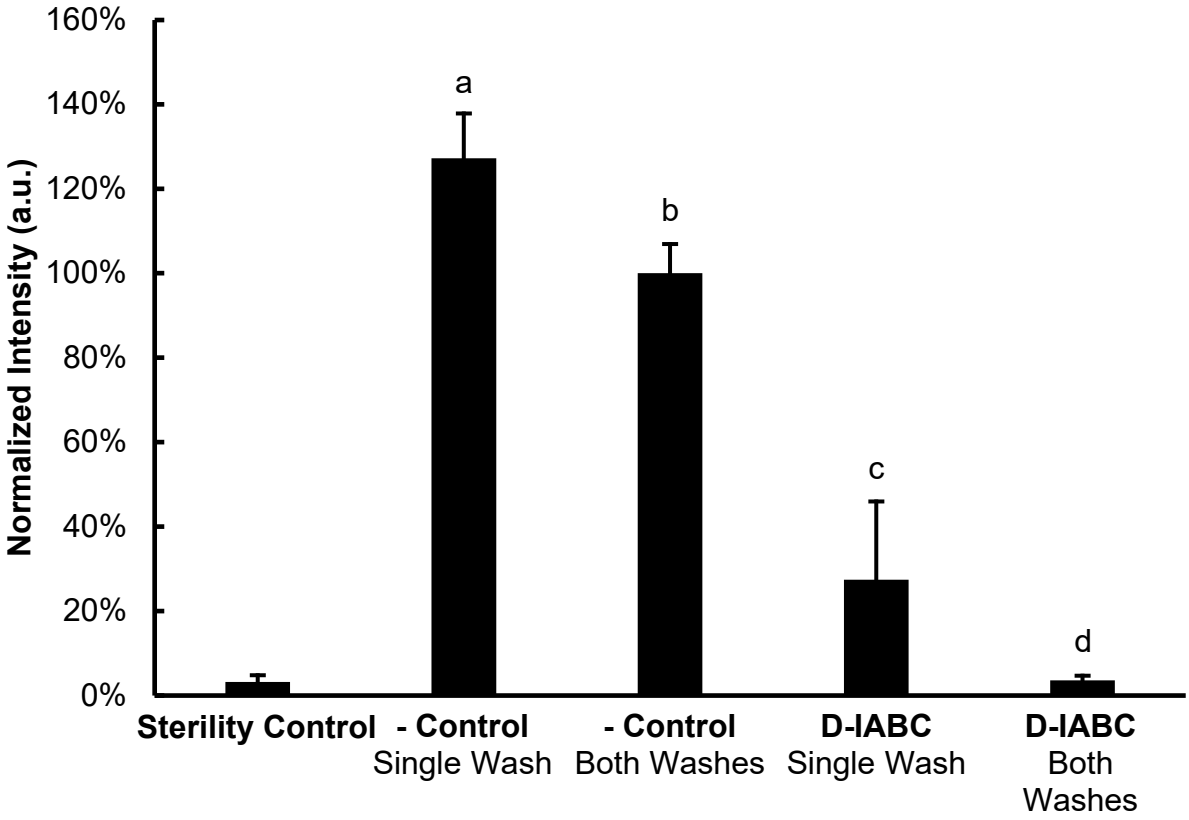

Figure 2.3 Comparison of updated and legacy crystal violet microplate anti-biofilm activity assay methods. 'Single Wash' indicates the legacy method employing wash steps only after staining with crystal violet. 'Both Washes' indicates the updated method employing a wash prior to and after crystal violet staining. Biofilms treated with D-IABC that subsequently encounter both wash steps display significantly less remaining biofilm than those only washed after staining. DPBS was used as a negative control for treatment of biofilms. Different letters denote significance as determined by pairwise comparisons ($p < 0.05$, $n = 4$).

2.3.8 Statistical Analysis

Data are represented as mean ± standard deviation. All statistical analyses were performed using the open-source statistical language R v3.5.3. Treatment groups were normalized to wells with bacteria treated with vehicle buffer (100%) to represent biofilm biomass percentages. Significance was denoted by letters as determined by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference post-test. Comparisons of means of two groups was performed by Student's t-test unless heteroscedastic data was observed (as determined by Bartlett's test) in which case Welch's t-test was performed. P values from comparisons that were less than or equal to 0.05 were considered statistically significant.

Results

2.3.9 P. pallidum D-IABC Degrades S. epidermidis Biofilm EPS

To obtain *P. pallidum* D-IABC, *S. epidermidis* biofilms were grown on porous polycarbonate membranes and exposed to *P. pallidum* to initiate biofilm predation. During biofilm predation, D-IABC were secreted and retained within the polycarbonate membrane. To evaluate the antimicrobial activity of D-IABC against *S. epidermidis* biofilms, a newly prepared biofilm was introduced to the D-IABC laden membrane to allow for indirect transfer of soluble compounds to the Dictyostelid-naïve biofilm (Figure 2.1). D-IABC produced from both *S. epidermidis* AH2589 and AH2490 predation caused complete disruption of biofilm EPS of the respective *S. epidermidis* variants, allowing all macroscopically visible biomass to be gently washed from the membrane (Figure 2.4).

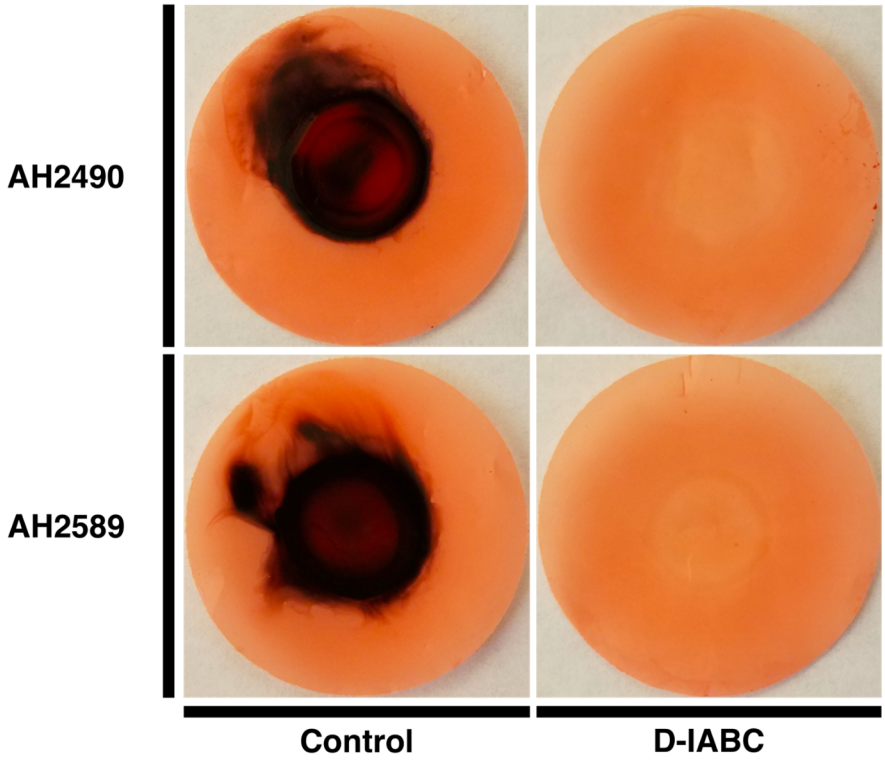

Figure 2.4 *P. pallidum* D-IABC produced during predation on *S. epidermidis* biofilms display robust anti-biofilm activity. AH2490 and AH2589 were grown on polycarbonate membranes to form biofilms and placed on membranes containing *P. pallidum* D-IABC during predation on *S. epidermidis* biofilms of their respective variants. After gently rinsing away liberated *S. epidermidis* cells, remaining cells were stained by application of the membrane to agar containing Congo Red dye as described in Methods. Membranes containing only *S. epidermidis* biofilm of the respective variant were placed under fresh biofilms as controls. D-IABC-treated biofilms of both AH2589 and AH2490 display complete, macroscopic disruption of *S. epidermidis* biofilm compared to their respective controls. Anti-biofilm activity was detected by treating biofilms with D-IABC in triplicate and singlicate for control biofilms. Representative images of each group are shown above.

Next, the recovered D-IABC was applied to *S. epidermidis* AH2490 biofilms for direct visualization of post-treatment membranes via SEM. As expected, bacteria that remained on the membrane after treatment lacked biofilm EPS between cells (Figure 2.5). By contrast, biofilm membranes treated with vehicle buffer solution exhibited substantial biofilm EPS between cells. For measuring anti-biofilm activity in a quantitative and high-throughput manner for the identification of active compounds within D-IABC fractions, a microplate-based biofilm biomass assay was adopted by employing crystal violet staining. Microplate *S. epidermidis* AH2490

biofilms displayed near-complete removal following the treatment of unfractionated D-IABC (Figure 2.6). The time required for disruption of membrane biofilms by D-IABC was also explored. As shown in Figure 2.7, disruption of membrane biofilms by D-IABC proceeded to completion in approximately two hours at 21°C.

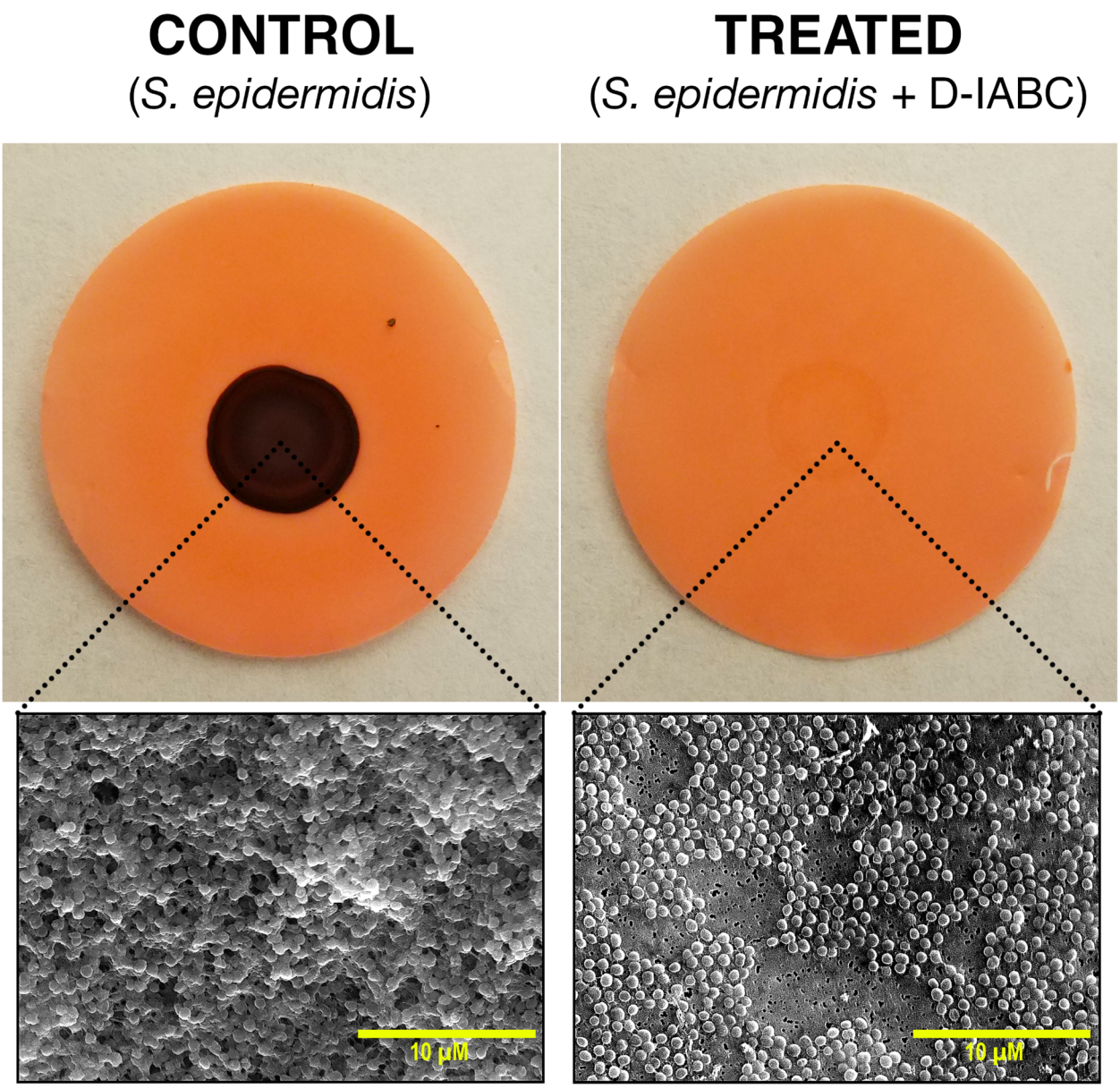

Figure 2.5 *S. epidermidis* biofilms visualized by scanning electron microscopy imaging following *P. pallidum* D-IABC treatment. Representative SEM images (below) of unstained biofilm treated overnight at 37°C with D-IABC display only residual membrane-adhered cells that lack biofilm EPS characteristic of *S. epidermidis* AH2490. Separate biofilms were treated identically to those imaged by SEM except for the SEM preparation process were stained with Congo Red (above) as described in Methods. The D-IABC buffer environment used in this experiment was pH 7.4 DPBS (exchanged by 10 kDa membrane hollow fiber dialysis) and pH 7.4 DPBS was used as a vehicle solution for the control.

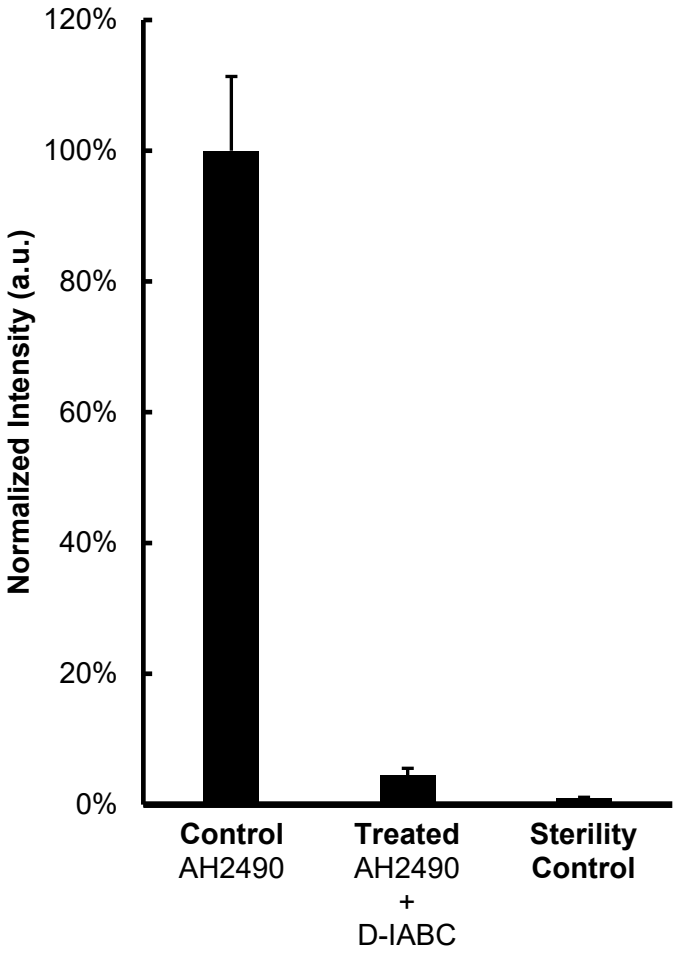

Figure 2.6 Quantitative removal of *S. epidermidis* AH2490 biofilm biomass by D-IABC in microplate format. Biomass was assessed by crystal violet staining following overnight treatment of D-IABC. Treated biofilms exhibit significantly less biofilm biomass than control as determined by an unpaired two-tailed Welch's *t*-test ($p < 0.01$, $n = 4$).

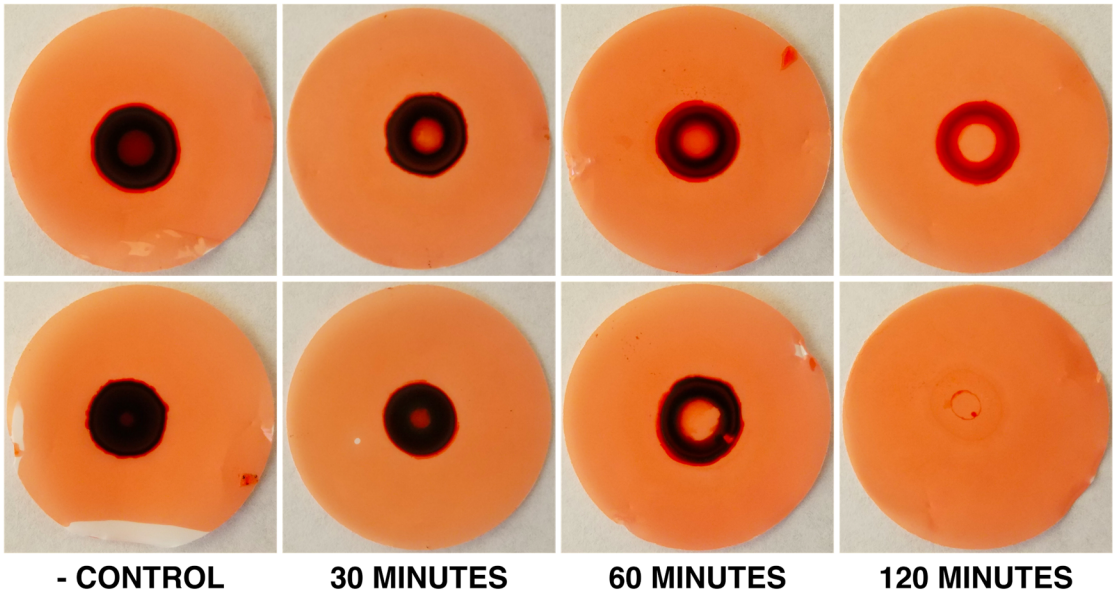

Figure 2.7 Time course of anti-biofilm activity against *S. epidermidis* AH2490 biofilms following D-IABC treatment at 21°C and pH 6.5. Separate biofilms were treated in duplicate at each timepoint and subsequently washed and stained as described in Methods.

To determine whether the predator-prey interactions between *S. epidermidis* and *P. pallidum* were required to produce D-IABC, the D-IABC isolation procedure was repeated in the absence of *P. pallidum*. Autoclaved *S. epidermidis* culture was applied to a diatomaceous earth filter cake and filtrate was collected to evaluate for anti-biofilm activity. In contrast to D-IABC, autoclaved *S. epidermidis* filtrate did not exhibit any anti-biofilm activity (Figure 2.8).

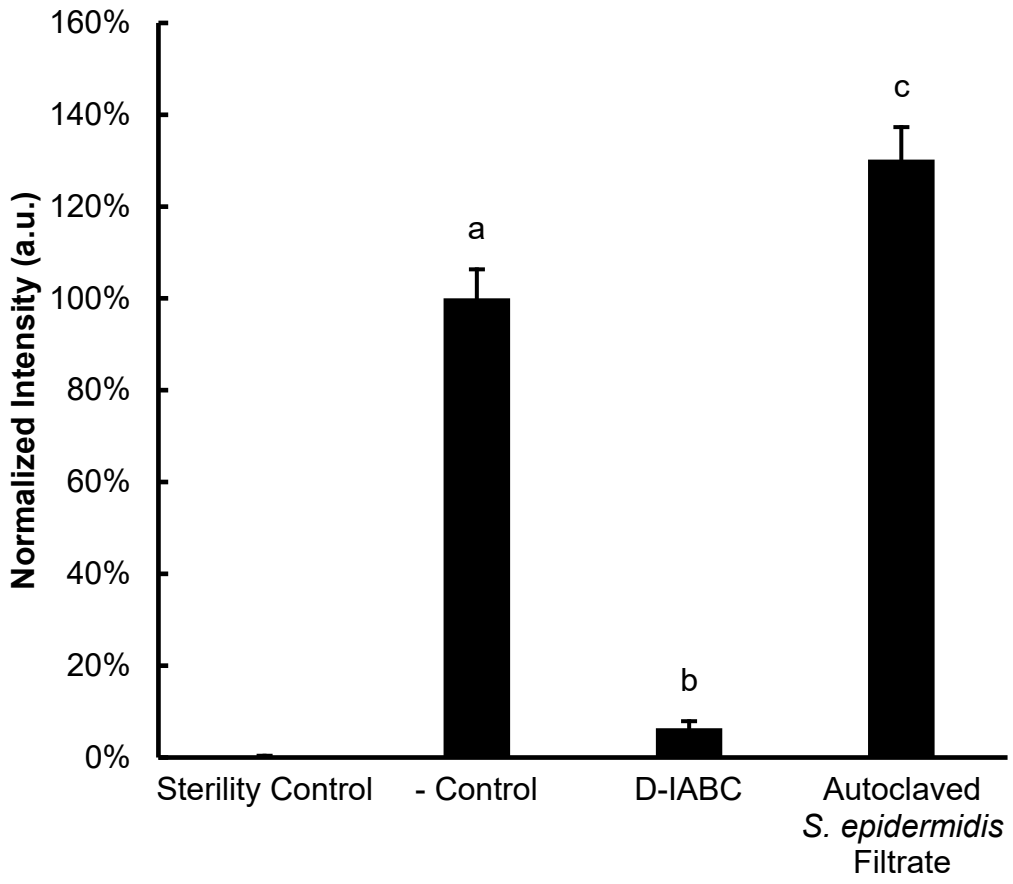

Figure 2.8 Quantitative comparison of *S. epidermidis* AH2490 biofilm biomass following D-IABC and autoclaved *S. epidermidis* filtrate treatment. Biomass was assessed by crystal violet staining following overnight treatment of D-IABC and autoclaved *S. epidermidis* filtrate. Data represent mean ± standard deviation. Different letters denote significance as determined by pairwise comparisons ($p < 0.05$, $n = 5$).

2.3.10 Size Fractionation of P. pallidum D-IABC

Degradation of biofilm EPS is a complex process potentially involving multiple enzymes or small molecules acting in concert to digest the multiple chemistries within EPS. To narrow the scope of the fractionation techniques used for further experiments, separation of small molecules from proteins and other macromolecules was sought first. A 3 kDa centrifugal membrane was used to facilitate the separation of these two classes within *P. pallidum* D-IABC prior to anti-biofilm activity

assays (Figure 2.2). Filtrate containing small molecules present within D-IABC fail to show any discernible activity against polycarbonate membrane-bound biofilms (Figure 2.9). Due to the reservoir feature of the 3 kDa Amicon centrifugal device, the filter retentate would be expected to also contain small molecules as well as concentrated macromolecules. This 'concentrate' of macromolecules, still including small molecules, maintains the ability to degrade biofilm EPS similar to unfractionated D-IABC used as a positive control (Figure 2.9).

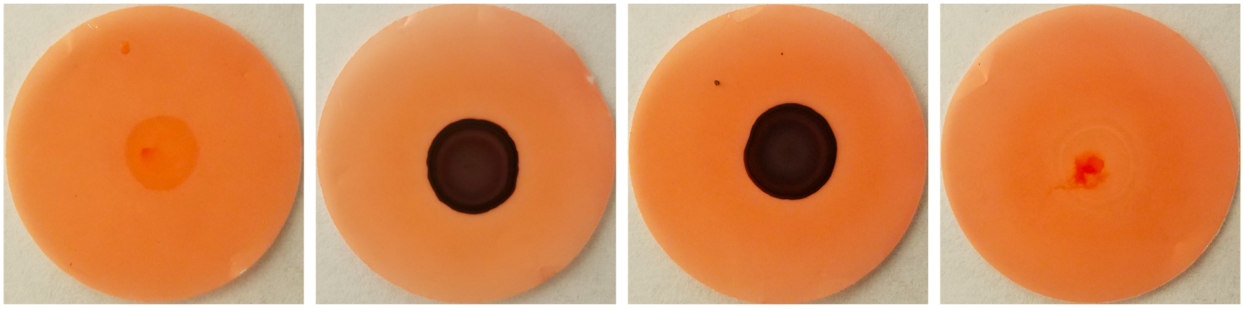

Figure 2.9 Anti-biofilm activity of *P. pallidum* D-IABC subjected to 3 kDa size fractionation against *S. epidermidis* biofilms. Concentrate material containing small molecules and macromolecules exhibited complete removal of *S. epidermidis* AH2490, and no discernable activity was observed in filtrate material containing small molecules alone. Unfractionated D-IABC was used as a positive control and liquid extracted from agar underneath a *S. epidermidis* AH2490 biofilm that did not receive *P. pallidum* spores was used as a negative control. Biofilms were washed and stained as described in Methods. Anti-biofilm activity was detected by treating biofilms in duplicate and representative images of each group are shown above.

2.4 Discussion

Recent demonstrations that Dictyostelids possess the ability to feed on biofilm-forming bacteria similarly to biofilm-deficient bacteria[9] have indicated that Dictyostelids may employ means to degrade biofilm EPS during predation. Robust anti-biofilm activity observed in the absence of physical contact with *S. epidermidis* prey cells indicates that this activity can be separated from the participating organisms (Figure 2.4). This observation suggests an exciting possibility that these anti-biofilm compounds can be produced or isolated for therapeutic or industrial use in combating infections that are intractable due to biofilm formation. Additionally, D-IABC ostensibly

removed all *S. epidermidis* biofilm EPS components as shown by electron microscopy in Figure 2.5, and only residual adherence to the polycarbonate surface remains with no EPS between cells or evidence of cell-cell adhesion. This suggests that D-IABC is capable of degrading EPS composed of multiple chemistries and may potentially involve multiple distinct compounds to carry out this activity. It is exciting to observe that cells enmeshed within the biofilm were completely released, as it is possible that released bacteria are no longer under low oxygen tension conditions found deep within biofilm and are now metabolically active. Cells that have resumed an active metabolic state would then forfeit their tolerance to antibiotics, indicating that potential adjuvant therapies combining antibacterial agents and D-IABC may be possible.

Time to full disruption is rapid and occurs in an acceptably short period of time if it were to be used in a treatment context (Figure 2.7). However, given theoretical limits regarding the time for bacteria to change protein expression,[20] it is still possible that anti-biofilm compounds that act directly on the biofilm EPS could be expressed by substrate *S. epidermidis* biofilms used in anti-biofilm activity assays. Therefore, the rapidity of anti-biofilm activity cannot exclude that *S. epidermidis* produces the anti-biofilm agent in cases where D-IABC was produced using sterilized *S. epidermidis* cells fed to *P. pallidum*. To preclude any speculation that sterilization of bacteria fed to *P. pallidum* may produce anti-biofilm activity observed in these experiments, sterilized *S. epidermidis* was prepared similarly to how D-IABC was produced by filteraid filtration in section 2.3.3 with the exception that no *P. pallidum* spores were added (Figure 2.8). Observations that this scenario, as well as liquid recovered from agar from beneath *S. epidermidis*-only polycarbonate membranes as used in negative controls in Figure 2.1 and Figure 2.4, does not produce anti-biofilm activity indicates that the presence of *P. pallidum* is strictly required. Consequently, *P. pallidum* likely produces these compounds as a response involved in its predation of bacteria. Although this seems to indicate that *P. pallidum* is responsible for the production of the active compound in D-IABC, it should be noted that it is also possible that *P.*

pallidum modifies or otherwise activates a constitutively expressed *S. epidermidis* compound whose activity survives the autoclave sterilization procedure.

Size fractionation of D-IABC in Figure 2.9 indicates that small molecules capable of passing through a 3 kDa membrane are not solely responsible for the observed anti-biofilm activity. Furthermore, concentrated macromolecules and remainder small molecules contained in the concentrate reservoir appear to retain full activity. It is important to note that small molecules such as cofactors may still be required for activity that is carried out in concert with proteins or other macromolecules, so it is possible that anti-biofilm activity could still be lost when small molecules are fully separated from macromolecule fractions. Even so, these results indicate that non-denaturing methods employed in protein fractionation may be of use to identify potential candidate proteins associated with anti-biofilm activity.

2.5 Conclusions

Here we have shown that *P. pallidum* produces soluble, macromolecular anti-biofilm compounds capable of robust degradation of biofilm EPS. Additionally, the presence of *P. pallidum* appears to be strictly required for the anti-biofilm activity to be observed. Although it may seem likely that *P. pallidum* produces the compound or compounds responsible for direct action on biofilm EPS components, it should be noted that these observations do not preclude that *P. pallidum* manipulates a constitutively expressed *S. epidermidis* protein to disrupt *S. epidermidis* biofilms. Fractionation and subsequent identification of candidate proteins associated with anti-biofilm fractions via matching to *S. epidermidis* and *P. pallidum* proteomes may be warranted given that macromolecule compounds are involved.

2.6 References

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CHAPTER 3. ISOLATION AND IDENTIFICATION OF PROTEIN RESPONSIBLE FOR D-IABC BIOFILM DISRUPTION

3.1 Abstract

Dictyostelids secrete proteins at multiple stages of their lifecycle, including predation. It is reasonable to suspect that Dictyostelids also secrete proteins during spore germination and during feeding on bacteria to assist with predation. Proteins potentially involved in disruption of *S. epidermidis* biofilm EPS were fractionated and fractions and native PAGE bands associated with anti-biofilm activity were submitted for proteome matching via mass spectrometry. All bands from active chromatographic fractions that harbored significant anti-biofilm activity against *S. epidermidis* biofilms contained high abundances of ornithine carbamoyltransferase with high similarity to sequences found in *S. epidermidis* proteomes. Although unexpected, *S. epidermidis* carbamoyltransferase may have unknown biofilm breakdown activity and may be constitutively expressed in *S. epidermidis* biofilms where it is susceptible to modification by *P. pallidum* for the purposes of predation.

3.2 Introduction

Multiple studies describe protein secretions by Dictyostelids during their lifecycle such as the proteins secreted during the stages of sporulation.[1] Given that Dictyostelid spores germinate in response to favorable conditions and the detection of bacterial prey,[2] it would not be unexpected that they secrete proteins that assist with predatory functions during germination as well as post-germination feeding. Dictyostelids are soil-dwelling organisms and their bacterial prey are expected to primarily exist as biofilms in soil environments.[3] Congruent with these expectations, it would follow that Dictyostelids secrete proteins capable of enzymatic breakdown of biofilm EPS chemistries or secrete proteins that initiate the production or activation of them.

Chapter 2 provided evidence that the combination of *P. pallidum* spores and *S. epidermidis* cells produced soluble macromolecules capable of disrupting *S. epidermidis* biofilms. Presumably, the identity of these macromolecules are proteins produced by *P. pallidum* that act in concert on biofilm EPS. However, constitutively expressed *S. epidermidis* zymogens or otherwise inactive proteins may also be activated by *P. pallidum* compounds to cause *S. epidermidis* biofilm autodegradation. In the present chapter we isolate and identify candidate compounds that may be responsible for anti-biofilm activity against *S. epidermidis*.

3.3 Methods

3.3.1 Protein Fractionation by Ion Exchange Chromatography

D-IABC produced by *P. pallidum* feeding on *S. epidermidis* biofilm were loaded onto Q Sepharose Fast Flow anion exchange resin (Cytiva, Marlborough, Massachusetts, United States, Catalog # 17051010) in a column format using an AKTA Start bench-top chromatography system (Cytiva, Marlborough, Massachusetts, United States, Catalog # 29022094) while collecting breakthrough protein to determine the level of D-IABC that did not bind to the resin. Protein content was monitored by an in-line UV detector measuring post-column absorbance at 280 nm. Sodium chloride (VWR, Radnor, Pennsylvania, United States, Catalog # JT3627-5) washes were employed to elute proteins from the resins by charge intensity.

3.3.2 Native PAGE Recovery of Band Activity via Electroelution

Bands were excised from an unstained, 4-16% polyacrylamide native PAGE gel (Thermo Scientific, Waltham, Massachusetts, United States, Catalog # BN1002) employing a Coomassie G-250 dye-protein interaction to confer negative charge to proteins for electrophoresis. A matching stained gel was used as a reference for the location of low protein-abundance bands in the unstained gel not evident by residual accumulation of the Coomassie G-250 dye present in

the running buffer (Thermo Scientific, Waltham, Massachusetts, United States, Catalog # BN2001 and Catalog # BN2002) on proteins after rinsing. Excised bands were individually minced with a scalpel blade and placed in a Float-A-Lyzer (Repligen, Waltham, Massachusetts, United States, Catalog # G235031) dialysis chamber with a 10 kDa membrane filled with native PAGE light cathode buffer. The chamber was placed in an electrophoresis tank for electroelution of proteins from the minced gel fragments in light cathode buffer at 200 volts for 20 minutes. Dialysis chambers containing eluted proteins and light cathode buffer were then transferred to an excess volume of ice-chilled Dulbecco's phosphate buffered saline (DPBS) (Fisher Scientific, Pittsburgh, United States, Catalog # AAJ61917AP) for overnight dialysis. After dialysis, minced gel fragments and protein solution were filtered by Vivaclear 0.8 µm centrifugal filters (Sartorius, Göttingen, Germany, Catalog # VK01P042) at 14,000 RCF to remove gel fragments. Filtrate was transferred to 3 kDa Amicon centrifugal filter devices (EMD Millipore, Burlington, Massachusetts, United States, Catalog # UFC500308) and centrifuged at 14,000 RCF for 30 minutes to concentrate protein and then diluted with DPBS to 500 µL and centrifuged again before bringing samples to final volumes of 250µL with additional DPBS before use in microplate anti-biofilm activity assays.

3.3.3 Mass Spectrometry Protein Identification

Bands to be submitted for analysis were stained with Instant Blue (Expedeon, Cambridge, United Kingdom, Catalog # ISB1L) for 60 minutes. Bands were digested overnight using sequencing grade modified trypsin (Promega, Madison, Wisconsin, United States, Catalog # V511A). After digestion, extracted peptides were dried and reconstituted in 20 µL Optima grade water with 0.1% formic acid and injected in volumes according to band staining intensity with the Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, Massachusetts, United States, Catalog # IQLAAEGAAPFALGMBDK). Separation was carried out with a 50-minute increasing acetonitrile gradient. To reduce sample carryover, 50-minute blanks were used between samples. 200 fmol of yeast Enolase was run as a control to verify performance of mass

spectrometer and nanoAcquity UPLC. Identification of most likely proteins from *S. epidermidis* and *P. pallidum* proteomes were determined by Proteome Discoverer 2.2 (Thermo Scientific, Waltham, Massachusetts, United States).

3.3.4 Statistical Analysis

Data are represented as mean ± standard deviation. All statistical analyses were performed using the open-source statistical language R v3.5.3. Treatment groups were normalized to wells with bacteria treated with vehicle buffer (100%) to represent biofilm biomass percentages. Significance was denoted by letters as determined by one-way ANOVA with Tukey's honestly significant difference post-test. Comparisons of means of two groups was performed by Student's t-test unless heteroscedastic data was observed (as determined by Bartlett's test) in which case Welch's t-test was performed. P values from comparisons that were less than or equal to 0.05 were considered statistically significant.

3.4 Results

3.4.1 Chromatographic Fractionation of P. pallidum D-IABC

Once the requirement of macromolecules for anti-biofilm activity was established, we aimed to fractionate D-IABC by ion-exchange chromatography to narrow the protein candidates responsible for anti-biofilm activity. Unbound breakthrough proteins and bound proteins subsequently eluted from anion exchange resin by stepwise washes of increasing conductivity were collected while monitoring for protein content by a 280 nm post-column UV detector (Figure 3.1). Collected fractions were applied to microplate biofilms to assess their anti-biofilm activity and all fractions except breakthrough material exhibited significant activity as measured by crystal violet absorbance (Figure 3.2).

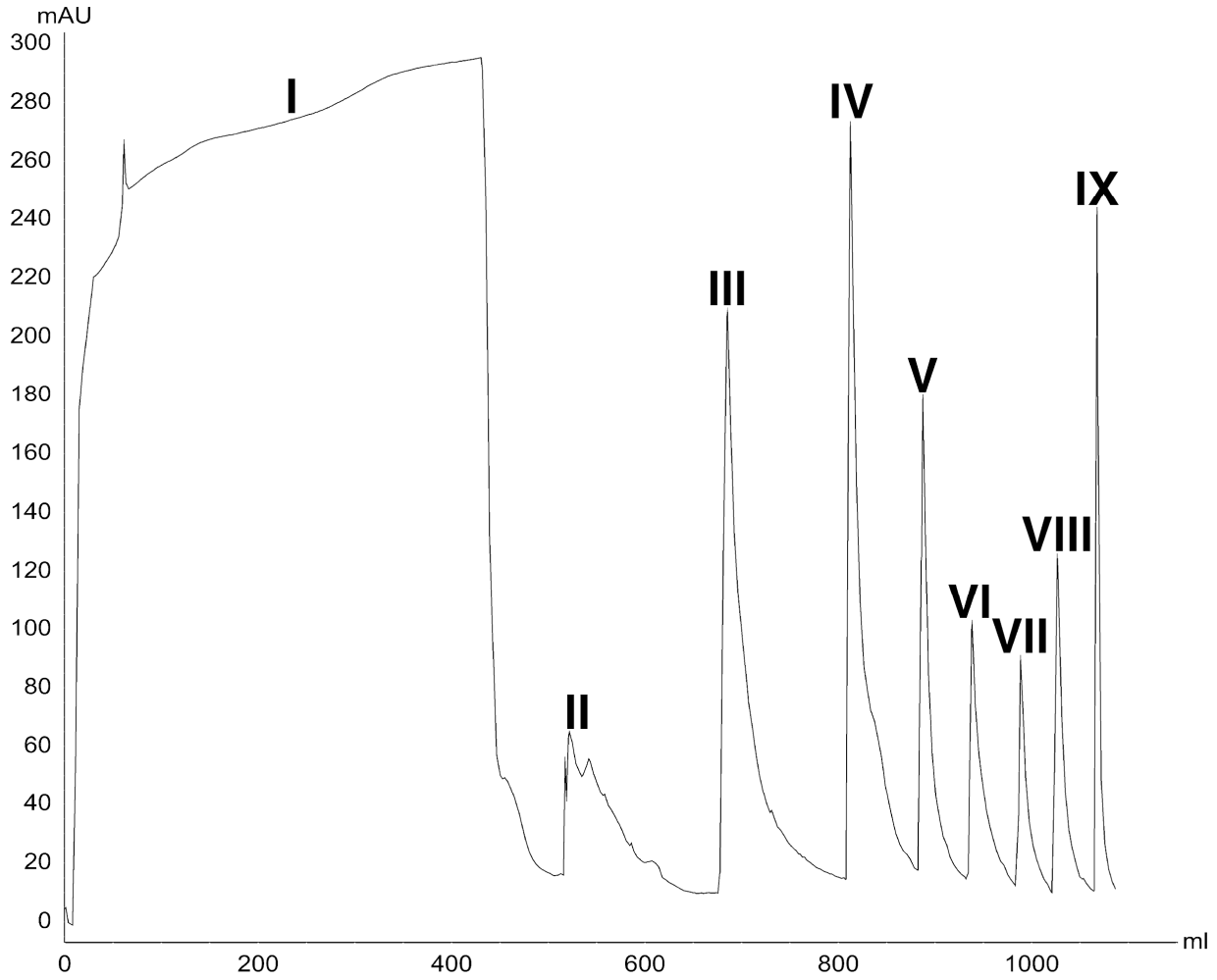

Figure 3.1 Ion exchange fractionation of anti-biofilm compounds produced by application of *P. pallidum* to *S. epidermidis* AH2490. Equilibration and wash buffer was 10 mM sodium phosphate at pH 6.5. Elution buffer conductivities were achieved by proportional mixing of equilibration buffer and 1 M NaCl. Post-column protein content was monitored by a UV detector at 280 nm. I) Unbound protein during application of anti-biofilm compounds, II) 50 mM NaCl, III) 150 mM NaCl, IV) 250 mM NaCl, V) 350 mM NaCl, VI) 450 mM NaCl, VII) 550 mM NaCl, VIII) 650 mM NaCl, IX) 1000 mM NaCl.

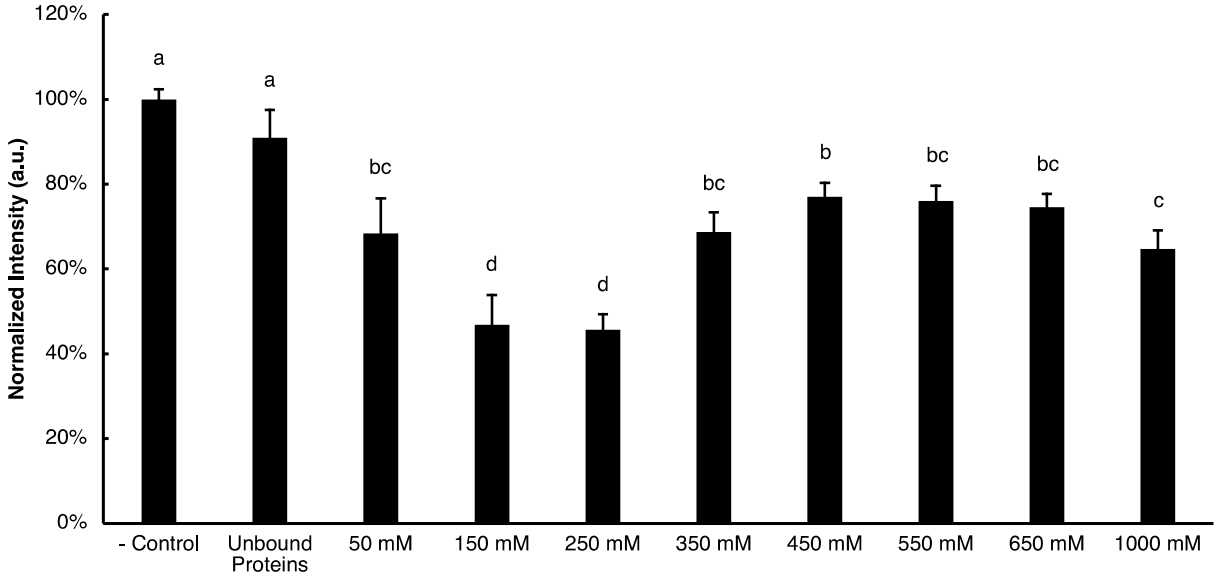

Figure 3.2 Anti-biofilm activity of fractions from ion exchange chromatography. Data represent mean ± standard deviation. Unbound proteins collected from the pooled breakthrough do not exhibit significant anti-biofilm activity. Substantial anti-biofilm activity is observed in 150 mM and 250 mM NaCl elutions and less intense but significant activity in all other elutions. 20 mM potassium phosphate at pH 6.5 was used as a negative control. Data from this figure was produced using the legacy version of the crystal violet microplate assay as indicated in Methods. Different letters denote significance as determined by pairwise comparisons ($p < 0.05$, $n = 4$).

3.4.2 Identification of Candidate Proteins Responsible for Anti-Biofilm Activity

The two fractions with the highest anti-biofilm activity from Figure 3B, 150 mM and 250 mM elutions, underwent further fractionation by native PAGE (Figure 3.3). Protein was recovered from these unstained native PAGE bands via electroelution and assayed for anti-biofilm activity by the crystal violet assay. Bands 1, 2, 4, and 5 from Figure 3.3 removed significant amounts of biofilm biomass as compared to a bovine thrombin band control treated in an identical manner (Figure 3.4). Stained native PAGE bands treated identically until staining were excised similarly and submitted for digestion and mass spectrometry for matching of protein sequences to *P. pallidum* and *S. epidermidis* proteome databases. Tables 1-4 indicate SEQUEST HT Uniprot_Staphylococcus epidermidis and Uniprot_Polysphondylium pallidum database matches

of sequences from submitted bands as well as common contaminant proteins such as human keratin and the porcine trypsin used for protein digestion.

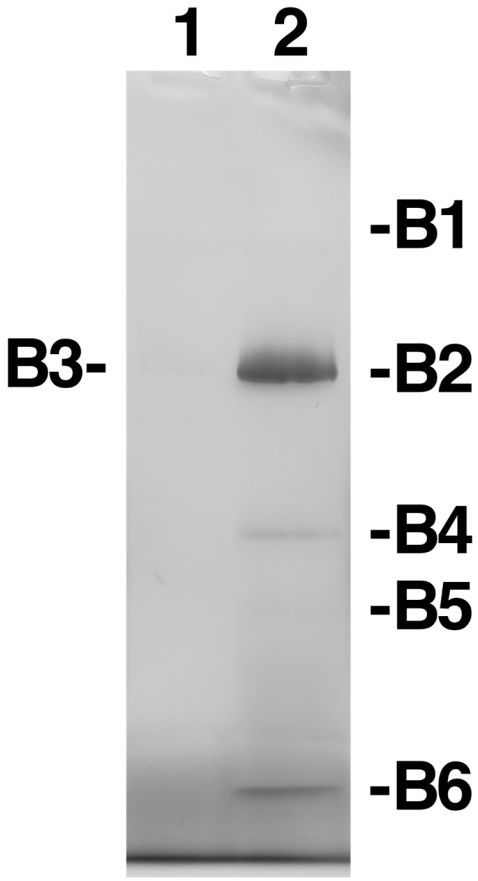

Figure 3.3 Native PAGE of 150 mM and 250 mM chromatography fractions. Only one weakly stained band was evident in the 150 mM chromatography fraction (lane 1) whereas several bands are apparent in the 250 mM fraction (lane 2). Bands excised from the gel for later electroelution and activity assays are labeled numerically.

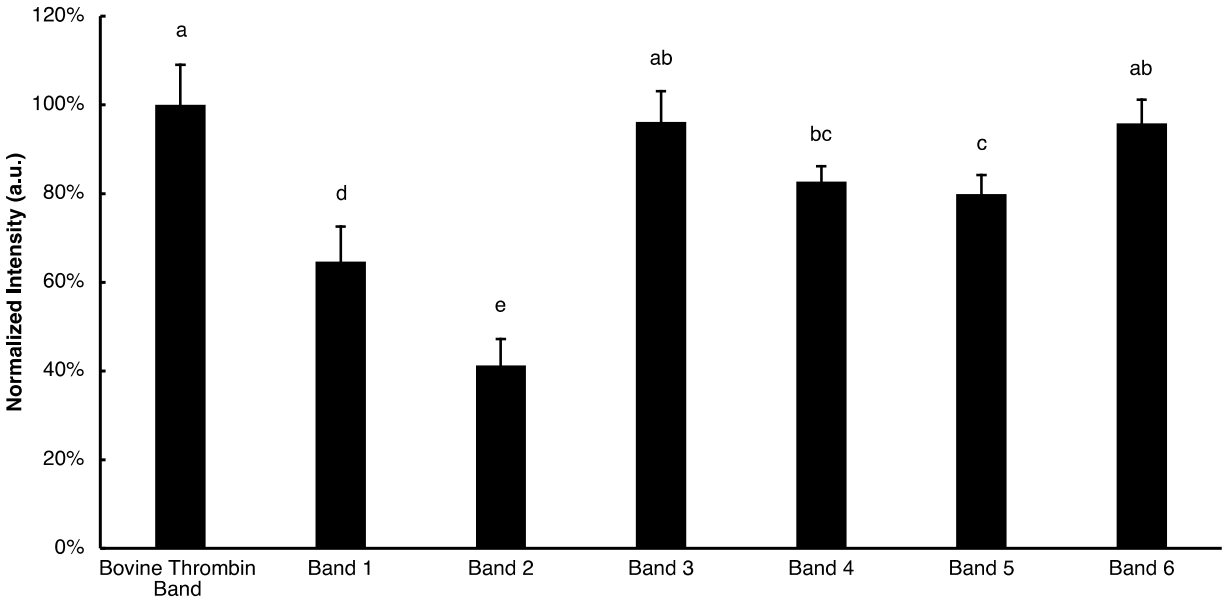

Figure 3.4 Anti-biofilm activity of native PAGE bands. Bands underwent an electroelution and dialysis procedure as described in Methods. Bands 1, 2, 4, and 5 exhibit significant anti-biofilm activity as compared to a bovine thrombin band excised from the native PAGE gel and prepared similarly as a negative control. Data represent mean ± standard deviation. Different letters denote significance as determined by pairwise comparisons ($p < 0.05$, $n = 4$).

Table 3.1 Identification of protein sequences from Band 1 by mass spectrometry.

Accession number	Description	Gene	Organism	Percentage of coverage	Amino acids	MW (kDa)	Peptides		
							Total	Spectrum matched	Unique
Q5HKJ4	Ornithine carbamoyltransferase	arcB-2	*S. epidermidis (ATCC 35984)*	61	333	37.5	19	184	19
Q5HKE8	Accumulation associated protein	aap	*S. epidermidis*	23	2397	255.5	6	24	6
Q5HM81	Dps family protein	SERP1748	*S. epidermidis*	22	148	22.7	2	9	2
Q5HNZ5	Superoxide dismutase [Mn/Fe]	sodA	*S. epidermidis*	17	199	16.8	2	7	2

Table 3.2 Identification of protein sequences from Band 2 by mass spectrometry.

Accession number	Description	Gene	Organism	Percentage of coverage	Amino acids	MW (kDa)	Peptides		
							Total	Spectrum matched	Unique
Q5HKJ4	Ornithine carbamoyltransferase	arcB-2	*S. epidermidis (ATCC 35984)*	89	333	37.5	84	1182	84
Q5HKE8	Accumulation associated protein	aap	*S. epidermidis*	21	2397	255.5	4	9	4
Q5HNZ5	Superoxide dismutase [Mn/Fe]	sodA	*S. epidermidis*	18	199	22.7	2	2	2
Q5HM81	Dps family protein	SERP1748	*S. epidermidis*	12	148	16.8	1	3	1
Q5HP05	Competence protein ComGA, putative	SERP1109	*S. epidermidis*	5	327	37.7	1	5	1
Q5HNI8	Septation ring formation regulator EzrA	ezrA	*S. epidermidis*	3	564	66.4	1	2	1
Q5HPQ2	Pyruvate ferredoxin oxidoreductase, alpha subunit	SERP0856	*S. epidermidis*	2	586	64.4	1	2	1

Table 3.3 Identification of protein sequences from Band 4 by mass spectrometry.

Accession number	Description	Gene	Organism	Percentage of coverage	Amino acids	MW (kDa)	Peptides		
							Total	Spectrum matched	Unique
Q5HM81	Dps family protein	SERP1748	*S. epidermidis (ATCC 35984)*	97	148	16.8	24	1056	24
Q5HP76	Nucleoside diphosphate kinase	ndk	*S. epidermidis*	56	149	16.7	6	41	6
Q5HKJ4	Ornithine carbamoyltransferase	arcB-2	*S. epidermidis*	43	333	37.5	9	59	9
Q5HNZ5	Superoxide dismutase [Mn/Fe]	sodA	*S. epidermidis*	43	199	22.7	5	12	5
Q5HQV1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gpmI	*S. epidermidis*	31	505	56.3	12	55	12
Q5HKE8	Accumulation associated protein	aap	*S. epidermidis*	21	2397	255.5	4	29	4
Q5HKM9	Lactonase drp35	Drp35	*S. epidermidis*	19	325	36.1	4	11	4
Q5HRX2	Inosine-5'-monophosphate dehydrogenase	guaB	*S. epidermidis*	17	488	52.6	7	36	7
Q5HR68	Flavohemoglobin	SERP0325	*S. epidermidis*	13	381	43.0	3	7	3
Q5HQ92	67 kDa Myosin-crossreactive antigen	SERP0663	*S. epidermidis*	4	591	67.7	2	7	2

Table 3.4 Identification of protein sequences from Band 5 by mass spectrometry.

Accession number	Description	Gene	Organism	Percentage of coverage	Amino acids	MW (kDa)	Peptides		
							Total	Spectrum matched	Unique
Q5HP76	Nucleoside diphosphate kinase	ndk	*S. epidermidis (ATCC 35984)*	84	149	16.7	16	169	16
Q5HKJ4	Ornithine carbamoyltransferase	arcB-2	*S. epidermidis*	67	333	37.5	22	102	21
Q5HM81	Dps family protein	SERP1748	*S. epidermidis*	66	148	16.8	6	46	6
Q5HNZ5	Superoxide dismutase [Mn/Fe]	sodA	*S. epidermidis*	55	199	22.7	15	45	15
Q5HKM9	Lactonase drp35	Drp35	*S. epidermidis*	50	325	36.1	18	81	18
Q5HR68	Flavohemoglobin	SERP0325	*S. epidermidis*	42	381	43.0	11	39	11
Q5HQV1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gpmI	*S. epidermidis*	39	505	56.3	17	39	17
Q5HQJ9	Glucose-6-phosphate isomerase	pgi	*S. epidermidis*	38	443	49.7	11	24	11
Q5HRF8	Putative heme-dependent peroxidase	SERP0235	*S. epidermidis*	33	249	29.5	6	16	6
Q5HMZ4	Aminotransferase, putative	SERP1481	*S. epidermidis*	31	429	48.9	11	21	11

3.5 Discussion

The observation that all non-contaminant proteins identified within active bands submitted for mass spectrometry identification match only to the *S. epidermidis* proteome adds a layer of complexity. It is possible that constitutively expressed *S. epidermidis* proteins, still active or able to be activated after autoclaving bacteria, or those produced by live *S. epidermidis* biofilms during activity testing are responsible for the final enzymatic activity related to degradation of biofilm EPS chemistries. However, compounds associated with the presence of *P. pallidum* must be at least peripherally active if not acting directly on biofilm EPS. This possibly indicates that another compound is produced by *P. pallidum* that interacts with *S. epidermidis* proteins to initiate their anti-biofilm activity. This would certainly not be the only case in nature where one organism manipulates the biochemistry of another during predation. Similar to how a protein within snake (*Echis carinatus*) venom cleaves prothrombin to form the fibrinogenase α-thrombin and cause blood coagulation in prey,[4,5] a constitutively expressed prey protein is activated to subvert a required physiological function whether that function is hemostasis or biofilm dispersion. This putative *P. pallidum* compound, with activity conserved throughout preparative dialysis or native-PAGE procedures in the experiments detailed in this work, may also have been a small molecule with low stability or high affinity for the *S. epidermidis* proteins identified such that most are protein-bound. Further molecular genetics studies are needed to identify major genes that provide a genetic basis for anti-biofilm activity of *P. pallidum*.

Among the six bands tested for *S. epidermidis* anti-biofilm activity, band 2 from Figure 3D exhibited the strongest anti-biofilm effects. Mass spectrometry analysis identified catabolic ornithine carbamoyltransferase (OCT) *arcB*, derived from *S. epidermidis*, as the top database match for band 2 (Table 3.2). Bands 1, 4, and 5, which display significant but lesser anti-biofilm activity as shown in Figure 3D, also contain OCT amongst the top matches (Tables 1, 3, 4),

possibly due to cross contamination between bands. Catabolic OCT (*arcB)* carries out the anaerobic degradation of arginine via phosphorolysis of citrulline.[6,7] Conversely, anabolic OCT (*argF*) is an enzyme that catalyzes the reaction between carbamoyl phosphate and L-ornithine in the anabolic biosynthesis of arginine in prokaryotes.[8] These proteins are generally recognized to be cytoplasmic in both prokaryotes and eukaryotes which confuses any direct involvement it might have regarding biofilm disruption. However, there have been reports in the literature indicating that OCT acts as a 'moonlighting' protein,[9] whereby a cytoplasmic protein has a secondary function outside of the cytoplasm, being either extracellular, membrane bound, or cell wall bound. OCT has been identified as a cell surface adhesin in *S. epidermidis*[10] and *S. aureus*.[11] In *S. aureus*, OCT is upregulated during biofilm formation and at biofilm maturity[6]. Furthermore, OCT has been characterized as an immunogenic cell surface-bound protein in *Streptococcus agalactiae*,[12] *Streptococcus pyogenes*,[13] *Streptococcus suis*,[14] and *Clostridium perfringens*.[15] In pre-treated *Streptococcus suis* cells, anti-OCT antiserum decreased bacterial adherence in a dose-dependent manner.[14] Considering that OCT has been shown to influence bacterial adherence and been identified as a cell surface-bound protein, it is plausible that OCT plays a role in anti-biofilm activity. The observed anti-biofilm activity could be achieved by modification of extracellular OCT by *P. pallidum* to induce activity responsible for biofilm disruption. Although the relation between OCT and anti-biofilm activity remains to be understood, these findings suggest that OCT may be a component of D-IABC responsible for anti-biofilm activity.

These experiments were limited in several aspects, most notably that it cannot be claimed for certain whether Dictyostelid products alone are capable of *S. epidermidis* biofilm disruption because all biofilms used for anti-biofilm activity testing involved those containing live bacteria. Specialized sterilization methods such as gamma irradiation to sterilize biofilms[16] without overwhelming denaturation of biofilm EPS would potentially allow for greater clarity with regard to the source and mechanism of anti-biofilm activity. Additionally, lack of genetic knockout studies

prevents conclusive determination of which genes or proteins are responsible for the observed biofilm disruption.

3.6 Conclusions

Several isolated D-IABC protein bands, notably the OCT dominant bands, possess significant anti-biofilm activity when recovered from native PAGE. Unexpectedly, all non-contaminant candidate proteins identified from mass spectrometry match proteins found within the *S. epidermidis* proteome. These observations may suggest that *P. pallidum* presumably induces *S. epidermidis* to express OCT to carry out anti-biofilm activity or modifies an inactive, constitutively expressed OCT to convert it to a biofilm-dispersing form. Although typically recognized as a cytosolic protein involved in cellular nitrogen metabolism, OCT is a moonlighting protein with recognized history of extracellular function concerning bacterial adherence similar to other biofilm-related *S. epidermidis* proteins. To the author's knowledge, this is the first known account of Dictyostelids manipulating bacteria to produce autocrine anti-biofilm compounds, ostensibly for the purposes of predation. These results add to a growing body of evidence that OCT performs functions beyond those related to nitrogen metabolism in prokaryotes.

3.7 References

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CHAPTER 4. POTENTIATION OF ANTIBIOTIC EFFICACY AND IMMUNE CLEARANCE BY D-IABC

4.1 Abstract

Application of anti-biofilm agents as adjuvants for antibiotic potentiation has received cursory attention despite its potential to combat biofilm-associated antibiotic tolerance understood to be nearly universal to bacterial species. Compounds used by Dictyostelids capable of disrupting biofilms during predation may be able to serve as appropriate anti-biofilm adjuvants that allow increased efficacy of antibiotics or immune clearance by host immune cells. Although Dictyostelid compounds provide a modest but significant potentiation of macrophage biofilm biomass removal, partial disruption of *S. epidermidis* biofilms with D-IABC does not re-sensitize macrophage NF-κB activation. While Dictyostelid compounds may themselves manipulate bacterial metabolic activity to produce anti-biofilm effects, methods used to evaluate antibiotic potentiation via metabolic activity may be insufficient to evaluate advantages of using Dictyostelid compounds to potentiate antibiotic activity.

4.2 Introduction

Although far from fully explored, several examples of adjuvant use of anti-biofilm agents for potentiation of antibiotics have been previously documented.[1-4] However, many anti-biofilm agents employed in this manner are used to prevent biofilm formation rather than disrupt already-formed biofilms. Anti-biofilm agents that are capable of disrupting biofilms after formation represent a potential therapy for biofilm-related infections that likely comprise most of the impact relating to morbidity, mortality, and economic burden.

Because antibiotic tolerance is mediated largely by dormancy of bacteria within the biofilm, examining Dictyostelid anti-biofilm products' abilities to sensitize bacteria to antibiotics is an alluring prospect. Additionally, inhibition of host immune responses is also observed to occur in response to *S. epidermidis* biofilms, such as the inhibition of NF-κB, considered master regulator of innate immunity, in macrophages.[5] Dormant bacteria released from the biofilm EPS by an anti-biofilm agent would presumably assume a planktonic state and therefore be susceptible to antibiotics whose mode of action often requires bacterial growth or metabolic activity as well as removal by host innate immune responses. As demonstrated in previous chapters, *P. pallidum* possesses strong antibiofilm activity against the *S. epidermidis* strains used, and numerous bacterivore Dictyostelids ostensibly harbor similar activity against many bacterial species. The aim of the experiments in this chapter were to determine if similar adjuvant use of D-IABC improves the efficacy of antibiotics or biofilm biomass clearance by macrophages.

4.3 Methods

4.3.1 Generation of P. pallidum D-IABC

D-IABC used for experiments described in this chapter was produced using methods identical to those described in section 2.3.3 relating to D-IABC produced for chromatographic purification except for the following deviations. *S. epidermidis* AH2490 culture was not autoclaved prior to application to diatomaceous earth. The final product was dialyzed into a pH 7.4 DPBS buffer environment by 10 kDa membrane hollow fiber dialysis (Sartorius, Göttingen, Germany, Catalog # SU01005DIS41L6).

4.3.2 D-IABC Potentiation of Vancomycin Efficacy on Microplate Biofilms

Microplate biofilms were formed as described in chapter 2 and media and was removed from biofilm wells before adding 50 µL of 3% (w/v) tryptic soy broth (Fisher Scientific, Pittsburgh, United States, Catalog # DF0370-17-3) containing 12 µM fluorescein diacetate (Sigma-Aldrich, St. Louis,

United States, Catalog # SBR00001) to each well. Subsequently, wells were treated with either D-IABC, 5 μg/mL vancomycin (Fisher Scientific, Pittsburgh, United States, Catalog # S25328A), both D-IABC and 5 μg/mL vancomycin, or vehicle solutions. Sterility control wells not containing bacterial biofilms were treated identically. Treated wells were then incubated at 37°C and well biofilm metabolism was monitored via deacetylation of fluorescein diacetate[6] by measurement of fluorescence at Ex 494 nm and Em 518 nm using a BioSpa 8 automated incubator and Cytation 5 multi-mode reader (Biotek, Winooski, Vermont, United States). Data were analyzed by a four-way repeated measures ANOVA using the R library rstatix v0.7.0. Analysis design was within-subjects with respect to time and between-subjects for treatment factors (presence or absence of bacteria, vancomycin, and D-IABC in microplate wells) to determine significance of type III test effects.

4.3.3 Treatments for Selective Removal of Macrophages from Biofilm

RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, Georgia, USA, Catalog # S12450), 100 U/mL penicillin-streptomycin (Gibco, Grand Island, NY, Catalog # 15140-122) before transfer to a microplate at 1000 cells/well. Following overnight incubation, the media was removed and macrophages were treated with 120 μL deionized H_2O or DPBS. Microplate biofilms formed as described in chapter 2 were treated similarly and no significant changes in mean biomass as measured by crystal violet staining were observed (Figure 4.1A). In contrast, the majority of macrophage-associated biomass was removed when these treatments were applied to plated macrophages (Figure 4.1B). Data were analyzed by performing Welch's t-test for Figure 4.1B due to unequal variation between treatments as determined by Bartlett's test ($p < 0.05$). Student's t-test was performed to analyze data from Figure 4.1A.

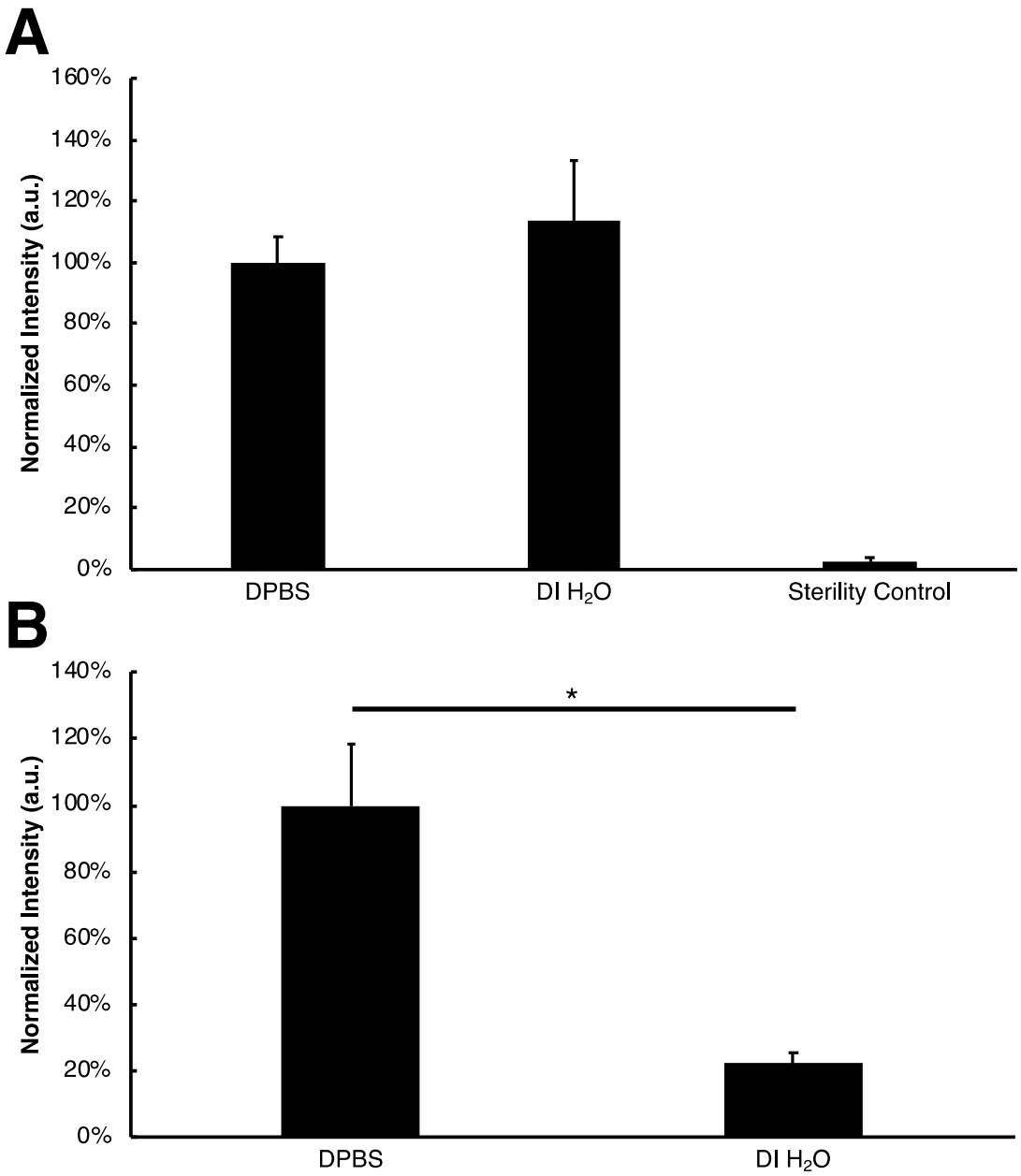

Figure 4.1 Treatments for selective removal of macrophages from biofilm. A) Crystal violet biomass staining of *S. epidermidis* microplate biofilms. Biofilms treated with deionized (DI) H_2O used for macrophage removal did not undergo biomass removal relative to DPBS used as a vehicle solution in microplate biofilm biomass experiments (n = 6). B) Crystal violet staining of plated macrophages. A majority of macrophage-associated biomass was removed from the plate surface via hypotonic lysis in the presence of DI H_2O (n = 4). *$p < 0.05$ compared to DPBS.

4.3.4 Macrophage Removal of Biofilm Biomass

S. epidermidis microplate biofilms, formed as described in chapter 2, underwent macrophage removal, disruption by D-IABC, macrophage removal and disruption by D-IABC, or DPBS vehicle treatment. Prior to treatment, bacterial media was removed from all wells and biofilms were washed with 240 µL DPBS. Macrophage removal of biofilm biomass was carried out by adding 1000 RAW 264.7 macrophages in 120 µL DPBS per well. Biofilm disruption was performed by addition of 120 µL of D-IABC. Vehicle wells and wells subjected to only one form of treatment were brought to a total volume of 240 µL by addition of DPBS. Following treatments of wells with their respective conditions for 3 hours at 37ºC, all wells were treated with DI H_2O suitable to selectively remove macrophages via hypotonic lysis as described above (Figure 4.1). Remaining biofilm biomass was determined by crystal violet staining as described in chapter 2 for determination of microplate biofilm biomass.

An additional experiment with an identical setup to that described above was performed with attenuated biofilms and diluted D-IABC. Attenuated biofilms were formed by 2-fold dilution of the inoculum bacteria during the addition of bacteria to microplate wells. D-IABC used in this experiment was diluted 768-fold to achieve a partially disrupted biofilm with remaining bacteria potentially susceptible to removal by macrophages (Figure 4.2).

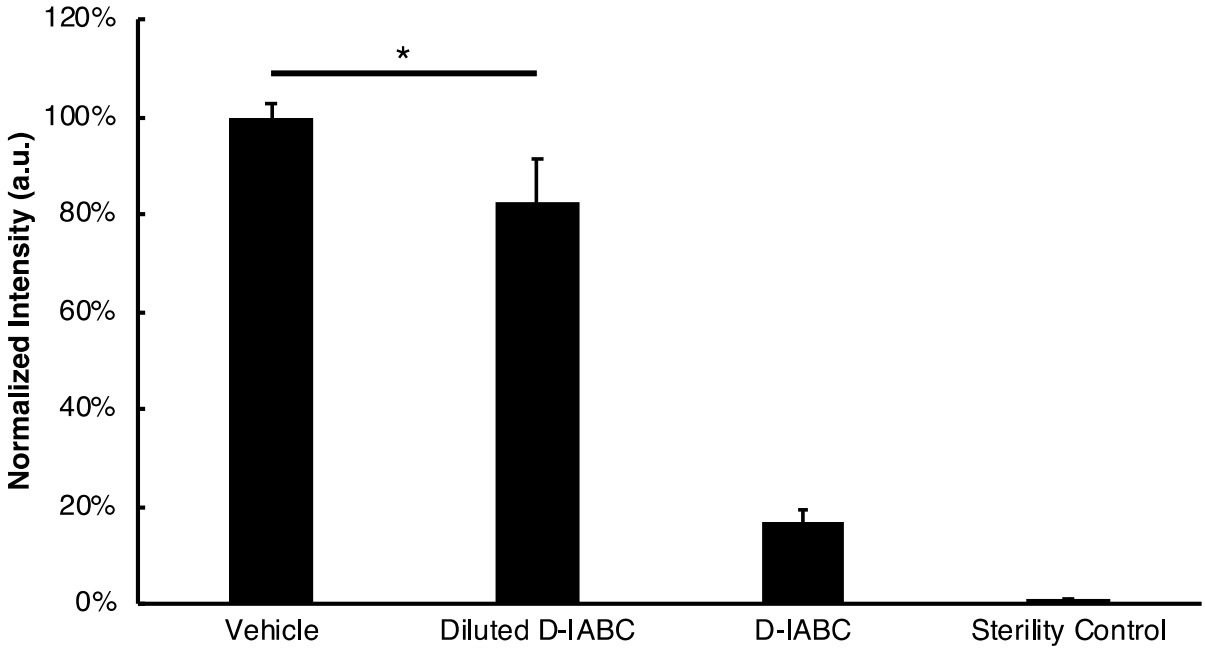

Figure 4.2 Dilution-attenuated biofilms and partially disrupted biofilms for macrophage experiments. Attenuated biofilms were formed by 2-fold dilution of the inoculum bacteria during the addition of bacteria to microplate wells. 'Diluted D-IABC' indicates that D-IABC was diluted 768-fold to achieve a partially disrupted biofilm (n = 6). *p < 0.05 for comparison shown.

4.3.5 D-IABC Potentiation of Macrophage NF-κB Activation on Microplate Biofilms

To measure the NF-κB activation of treated macrophages, a luminescent reporter macrophage cell line utilizing a plasmid containing *Renilla reniformis* luciferase under the transcriptional control of a NF-kB response element was used to produce luminescence in response to NF-κB activation. NF-κB Leeporter™ Luciferase Reporter-RAW 264.7 macrophages (Abeomics, San Diego, California, USA, Catalog # 14-100ACL) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin before being washed in DPBS and transferred in 100 µL of DPBS to select wells at 100,000 cells/well in a 96-well microplate (Corning, New York, United States, Catalog # 3917) after wells were treated for 4 hours with D-IABC or vehicle treatment. Macrophages or vehicle DPBS was incubated for 16 hours in wells containing biofilms, LPS (Sigma-Aldrich, St. Louis, United States, Catalog # L2630), vehicle, or a combination thereof. D-IABC used in this experiment was fractionated according to methods

described in section 3.3.1 to prevent confounding macrophage NF-κB activation by bacterial compounds present in unfractionated D-IABC that bound to the microplate well during the D-IABC treatment period (data not shown). Fractionated D-IABC was also diluted 96-fold to only partially disrupt dilution-attenuated biofilms that were formed in the microplate similar to methods described in section 4.3.4 and results from Figure 4.2. Following addition and incubation of macrophages in select wells at 37°C, luminescence was measured according to manufacturer instructions.

4.3.6 Statistical Analysis

Data are represented as mean ± standard deviation. All statistical analyses were performed using the open-source statistical language R v3.5.3. Treatment groups were normalized to wells with bacteria treated with vehicle buffer (100%) to represent biofilm biomass percentages. Significance was denoted by letters as determined by one-way ANOVA with Tukey's honestly significant difference post-test. Analysis of main effects and interaction effects in datasets without repeated measures was performed by two-way ANOVA. Analysis of main effects and interaction effects in datasets containing repeated measures was performed by four-way ANOVA using the R library rstatix v0.7.0. Comparisons of means of two groups was performed by Student's t-test unless heteroscedastic data was observed (as determined by Bartlett's test) in which case Welch's t-test was performed. P values from comparisons that were less than or equal to 0.05 were considered statistically significant.

4.4 Results

4.4.1 D-IABC Potentiation of Antibiotic Efficacy

Microplate biofilms in the presence of fluorescein diacetate were treated with vancomycin, D-IABC, vancomycin and D-IABC, or vehicle solutions to determine their individual impact on *S.*

epidermidis biofilm viability and interaction effects of vancomycin and D-IABC. Predictably, the presence of bacteria caused a significant increase in the deacetylation of fluorescein diacetate over time (bacteria:time interaction effect, $p<0.001$, Figure 4.3A). Additionally, the D-IABC main effect ($p<0.001$), D-IABC:time interaction effect ($p<0.001$), and D-IABC:bacteria interaction effect ($p<0.001$) accounted for a significant reduction in the deacetylation of fluorescein diacetate. The vancomycin main effect was significant ($p<0.001$), whereas the vancomycin:time, vancomycin:D-IABC, vancomycin:bacteria, and vancomycin:D-IABC:time interaction effects were not significant (Figure 4.3A-B).

After the experiment in Figure 4.3A-B was complete, biofilm biomass was measured by crystal violet assay as described in chapter 2 to ensure that D-IABC retained anti-biofilm activity in experimental conditions. As expected, biofilm wells treated with D-IABC showed significant reductions ($p<0.001$) in biofilm biomass and vancomycin-treated wells were not significantly different than their respective controls without vancomycin (Figure 4.3C).

Supernatant from microplate biofilm wells formed and treated identically to those in Figure 4.3A-B were plated on tryptic soy agar to determine counts of colony forming units (CFU) to assess effects of vancomycin on bacterial viability. Supernatant from wells treated with 5 μg/mL vancomycin produced significantly lower CFU counts than control containing vehicle solutions ($p<0.01$, Figure 4.3D).

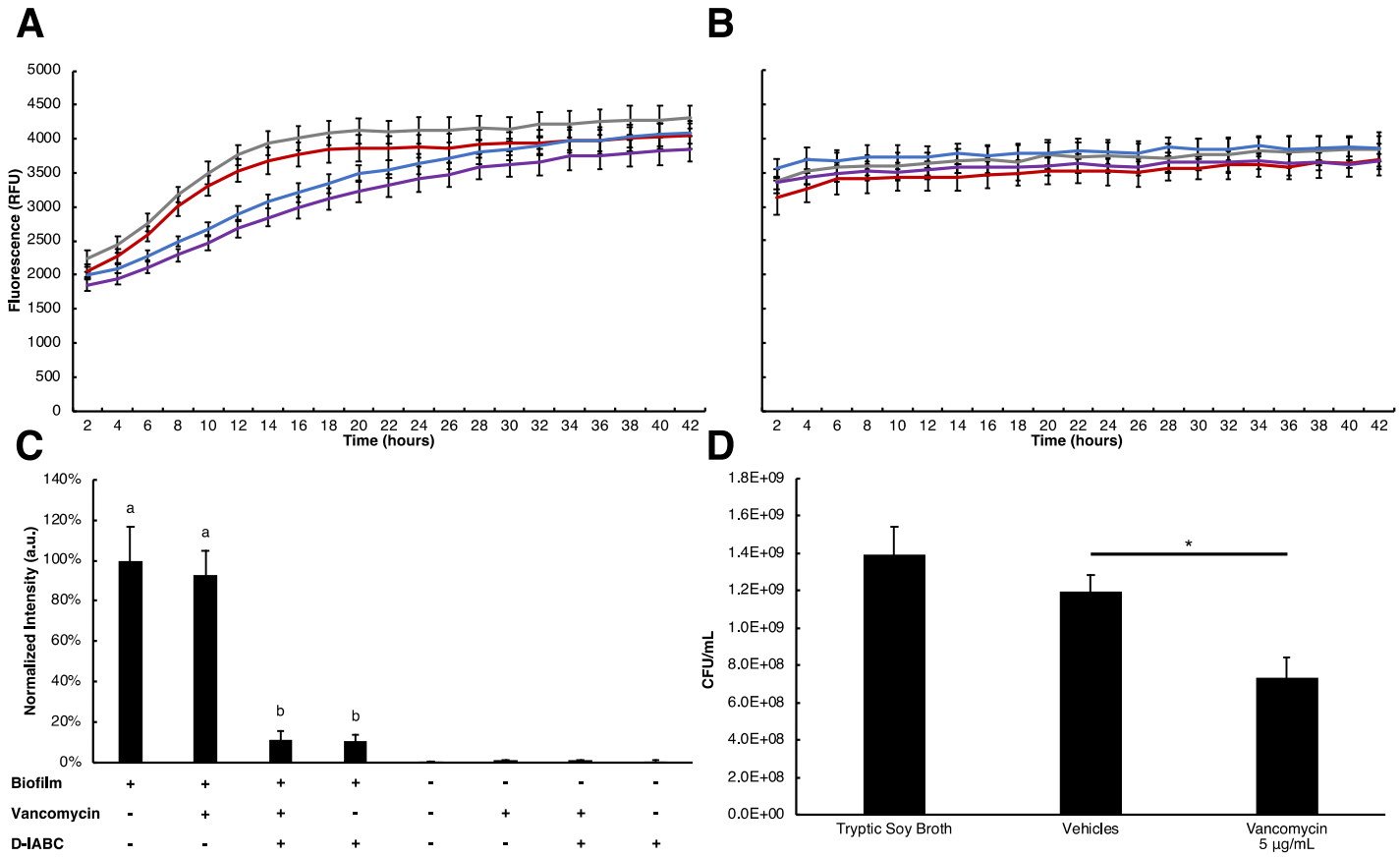

Figure 4.3 D-IABC potentiation of vancomycin efficacy on biofilm viability. A) Fluorescent quantification of biofilm viability in microplate biofilm wells exposed to 5 µg/mL vancomycin (red), D-IABC (blue), vancomycin and D-IABC (violet), or vehicle solutions (gray) (n = 12). B) Fluorescent quantification of biofilm viability in microplate sterility control wells exposed to 5 µg/mL vancomycin (red), D-IABC (blue), vancomycin and D-IABC (violet), or vehicle solutions (gray) (axes identical to A, n = 3). C) Final biofilm biomass as measured by crystal violet staining of wells from A (n = 12) and B (n = 3). Different letters denote significance as determined by pairwise comparisons ($p < 0.05$). D) Overnight treatment of microplate biofilm wells with vancomycin 5 µg/mL vancomycin followed by CFU plating of suspended culture *$p < 0.05$ for comparison shown.

4.4.2 D-IABC Potentiation of Macrophage Biofilm Biomass Removal

A modification of the methods used to form microplate biofilms was performed to allow greater sensitivity to observe macrophage biofilm biomass reduction effects in Figure 4.4. Without these modifications, the D-IABC treatment main effect is significant and macrophage treatment main effect and macrophage:D-IABC interaction effect are not significant (Figure 4.4A). D-IABC produced according to methods described in section 4.3.1 caused near complete removal of biofilm biomass at native concentrations and full-strength biofilms possibly contained excessive amounts bacteria, preventing observation of substantial proportional removal of biofilm biomass-associated crystal violet staining by macrophages. Therefore, an attenuated microplate biofilm and dilute D-IABC was used as described in section 4.3.4 to provide few enough bacteria to observe a reduction in overall biomass from macrophages and, in the case where biofilms were also treated with dilute D-IABC, a partially disrupted biofilm potentially susceptible to biomass reduction by macrophages as measured by crystal violet staining. Using this modified method, a significant interaction effect between D-IABC and macrophage treatments was observed as well as a significant D-IABC main effect on biofilm biomass reduction (Figure 4.4B-C).

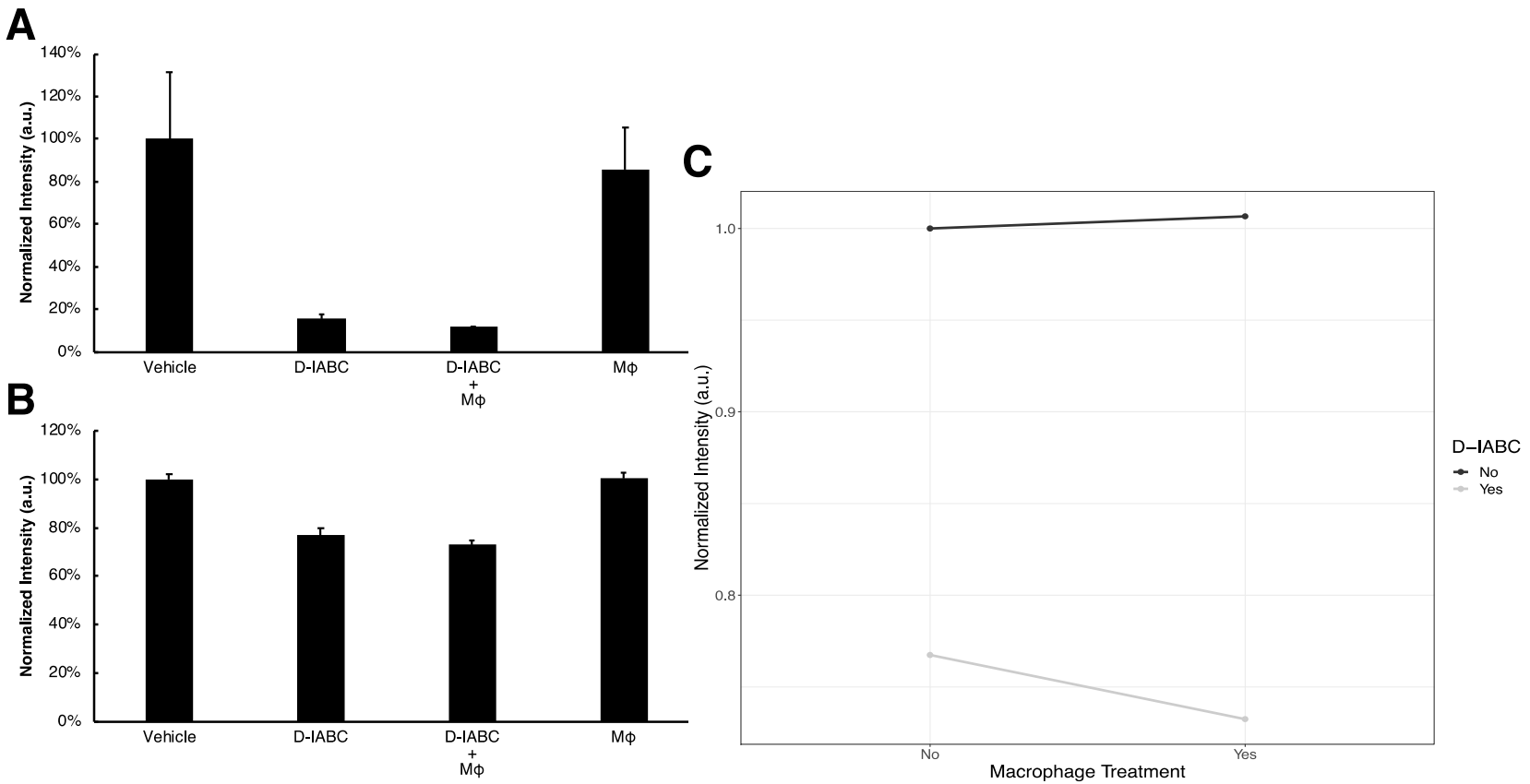

Figure 4.4 D-IABC potentiation of macrophage removal of biofilm biomass. A) Crystal violet assay of biofilms treated with D-IABC, macrophages (Mφ), or both (n = 6). D-IABC main effect was significant ($p < 0.05$). B) Crystal violet assay of attenuated biofilms treated with 768-fold diluted D-IABC, macrophages, or both (n = 6). D-IABC main effect and interaction effect of D-IABC and macrophage treatment were significant ($p < 0.05$). C) Interaction plot of B. Diluted D-IABC treatment of attenuated biofilms potentiated biofilm biomass removal of macrophages.

4.4.3 D-IABC Potentiation of Macrophage NF-κB Activation

Macrophage NF-κB activity was observed across wells containing naïve or partially disrupted biofilms formed by prior treatment with D-IABC, macrophages, or LPS as a positive control. Whether or not *S. epidermidis* biofilms were untreated or partially disrupted by D-IABC, all wells including biofilms failed to produce luminescence signal that was significantly higher the vehicle background irrespective of the inclusion of macrophages. Prior treatment of wells that did not contain biofilm with fractionated D-IABC did not cause a detectable increase NF-κB activation due to residual compounds bound to the well surface once macrophages were added to the well. In wells containing only macrophages and LPS as a positive control for NF-κB activation, luminescence signal was significantly increased. However, wells containing macrophages, naïve biofilms, and LPS do not exhibit activation significantly greater than the vehicle background signal (Figure 4.5).

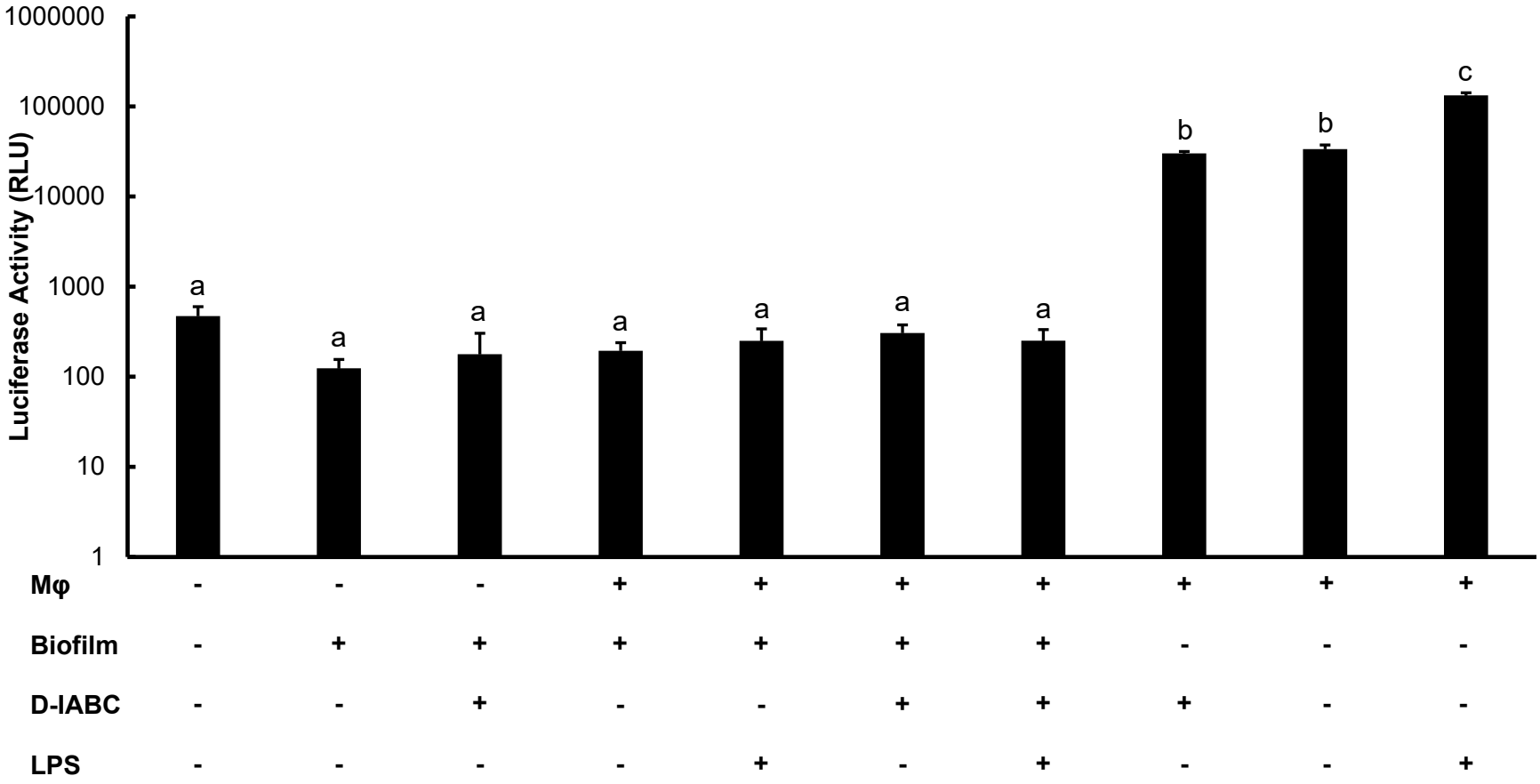

Figure 4.5 D-IABC potentiation of macrophage activation. Macrophage NF-κB activity measured in a cell line with a plasmid containing a luciferase-associated NF-κB response element was observed across wells containing naïve or partially disrupted biofilms formed by prior treatment with D-IABC, macrophages, or LPS as a positive control. All wells including *S. epidermidis* biofilms exhibited reduced luminescence signal compared to untreated macrophages, including the vehicle-only control. Wells containing residual fractionated D-IABC after prior treatment did not exhibit a significant difference from wells previously treated with vehicle to which only macrophages were added ($p = 0.9327$). Significantly increased luminescence signal was observed in wells containing macrophages treated with LPS as a positive control. Results are displayed on a logarithmic y-axis and different letters denote significance as determined by pairwise comparisons ($p < 0.05$, $n = 4$).

4.5 Discussion

4.5.1 Antibiotic Potentiation

The application of an anti-biofilm compound, even if not broad-spectrum, to return bacteria exhibiting antibiotic tolerance to a susceptible state via biofilm disruption is an exciting prospect; however, results shown here imply an unclear outcome. Significance of the bacteria:time effect indicates that sterility well effects do not account for over-time changes seen in wells containing bacteria as expected. D-IABC inhibited fluorescence generation, potentially due to metabolic inactivation or killing of bacteria. If the OCT protein is responsible, it is possible that substantial changes in extracellular levels of its substrate or product can affect the metabolic state of exposed bacteria.

Lack of significance of vancomycin:D-IABC, vancomycin:bacteria, and vancomycin:D-IABC:time interaction effects belie the expectation that synergistic effects of treating biofilms with vancomycin and DIABC together would be observed. Instead, the resulting outcomes show no evidence that bacterial metabolism has been altered beyond the sum of their individual treatment factor parts. Furthermore, failure to observe evidence of a vancomycin:bacteria interaction effect indicates insufficient evidence was found to differentiate an effect of vancomycin acting on the bacteria beyond that of the effect of vancomycin observed on the sterility wells. This suggests the possibility that this method, as performed, is not appropriate to determine differences in metabolism when vancomycin or its DMSO formulation is used to kill bacteria. Given that the concentration of vancomycin used in Figure 4.3 is sufficient to cause a significant reduction in CFU counts after plating, vancomycin may have an inhibitory effect on fluorescein diacetate deacetylation in some way. Additionally, the use of a nucleophilic, phosphate-based buffer creates a possible confounding effect of background phosphate-derived deacetylation of the probe,[6,7] where any treatment effect measured in this experiment could have been a contribution of

treatments muting or exacerbating this effect rather than having their own direct effect on bacterial metabolism or viability. Similar experimental designs, specifically those without repeated measures, could also be confounded by potential occlusion of fluorescent signal in bacteria- and biofilm-containing wells when comparing results to sterility well controls.

4.5.2 Macrophage Potentiation

The principal purpose of the experiments in Figure 4.4 was to determine the presence of an interaction effect between the D-IABC and macrophage treatments. Results indicate that it may exist, potentially due to a synergistic effect provided by D-IABC removal or disruption of biofilm EPS, potentially allowing phagocytosis of bacteria that no longer contribute to stained biomass after hypotonic lysis that removes macrophage-associated crystal violet staining. This may also have been due to preventing biofilm EPS-related inhibition of activities upstream of macrophage phagocytosis, such as activation of NF-κB.[5] However, these results could also be only, or in addition to, macrophage-related degradation of the matrix that is otherwise inhibited when D-IABC is not applied because crystal violet also strongly stains the polysaccharide components of the biofilm EPS. Although the interaction effect is significant, the difference in means is quite small. Therefore, other methods measuring activation of macrophages or phagocytic activity may be more suitable. It is also possible that D-IABC limits or reduces the residual staining left by macrophages, potentially introducing a confounding effect that prevents proper estimation of this D-IABC:macrophage interaction effect.

These results suggest potential future benefits of using D-IABC for treatment of biofilm-related infections, but the *in vitro* system employed here is markedly different from a true *in vivo* environment. Adding mouse macrophages to microplate biofilms does not accurately represent the host innate immune response, let alone the adaptive immune response involved in biofilm wound infections. Additionally, the methods employed here only concern bacteria still embedded

in a partially disrupted biofilm and not the cells released from it as would be present in a treated wound. Therefore, these findings could be used as justification for the use of animal wound models in measuring host macrophage activation or wound bioburden in future studies. Electron microscopy may be useful to rule out potential confounding effects that could have been present while measuring biofilm biomass reduction interaction effects between D-IABC and macrophage treatments. Direct visualization of activated and non-activated macrophages may confirm the mechanism involved.

Considering the macrophage NF-κB activation results, the mechanism of biofilm biomass removal as indicated by reduced staining in Figure 4.4B may be either independent of macrophage activation or severely blunted due to prevention of NF-κB activation if dependent on triggering of the innate immune response functions. Although it cannot be entirely excluded that the macrophages were killed after application to biofilms, similar experiments in the literature employing mouse macrophages find no changes in viability of macrophages added to biofilms compared to controls.[5] However, the authors of that article declined to show the trypan blue results in their publication. Prevention of macrophage NF-κB activation by *S. epidermidis* is also reported by the authors as well as others in the literature for *S. aureus*, so observation of this effect is certainly not without precedence.[5,8] The observation of abrogated NF-κB activity in macrophages added to biofilm in the presence of LPS in Figure 4.5 grants a substantial confounding effect to the experimental design. Presumably, biofilms that were only partially disrupted still contained factors responsible for NF-κB inactivation and would similarly blunt any potential re-sensitization of macrophages by prior treatment of biofilms with D-IABC. Interestingly, Schommer *et al.* report observations that mechanical dispersal of *S. epidermidis* biofilms partially, but not fully, restored NF-κB activation.[5] It may be possible that native concentrations of D-IABC are required which cause a full disruption of *S. epidermidis* biofilms utilized in this work in order to recover macrophage NF-κB activation. However, observations that even conditioned media

from the growth of *S. aureus* biofilms are also capable of inhibiting macrophage phagocytosis suggest that soluble compounds preventing NF-κB activation may still be present even after complete disruption of biofilms.[8]

4.6 Conclusions

This chapter describes the potential auxiliary use of D-IABC in the eradication of biofilm-encased bacteria. Although the value of antibiotic potentiation is unclear, other antibiotics or antibiotic formulations[7] should be compatible with the fluorescein diacetate metabolic assay for measurement of potentiation. Interestingly, D-IABC itself may have a strong impact on *S. epidermidis* viability or metabolism wherein OCT could be manipulating bacterial nitrogen metabolism from the extracellular space. There also exists some evidence that macrophages' ability to remove biofilm biomass benefits from D-IABC treatment. However, this mechanism may be independent from macrophage NF-κB activation or may occur before NF-κB activation is nearly fully inhibited by bacterial components. These effects could potentially be extrapolated to other aspects of host immune responses to biofilm infections for both innate and adaptive immunity and warrant further study.

4.7 References

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CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Many bacterial biofilm solutions are centered around the prevention of biofilm formation such as inhibition of quorum sensing that initiates the production of EPS components.[1] Because these treatments are often only successful in preventing formation of biofilms but not the disruption of already-formed biofilms, their efficacy is limited to less-relevant prophylactic use.[2] Due to this drawback, there exists a significant need for treatments able to remove already-formed biofilms present in biofilm-related infections. Anti-biofilm compounds that are capable of disrupting these biofilms have previously been discovered such as peptides mimicking those from the human immune system with broad-spectrum anti-biofilm activity.[3] However, few anti-biofilm drugs have been commercialized,[4,5] and, given the broad range of biofilm species that Dictyostelids can successfully disrupt, broad-spectrum anti-biofilm drugs could presumably be discovered by isolation of active compounds from Dictyostelid products. The discovery of nonantibiotic compounds with broad-spectrum anti-biofilm activity for the eradication of biofilm infections have been referred to as modern 'holy grails'.[6] Additionally, few accounts of human infections by Dictyostelids exist in the literature.[7] Because almost no Dictyostelids have been reported as being pathogenic to humans, it is tempting to speculate that their anti-biofilm agents may generally possess favorable safety profiles. Their potential use to re-sensitize biofilm-encased bacteria to antibiotics and immune clearance constitutes a large part of an exciting new class of drugs to treat intractable biofilm-related infections.

In this report, attention was focused on the thermotolerant and polyphagous Dictyostelid species *P. pallidum*[8] due to its suitability for producing proteins or small molecules active at human physiological temperatures that might have anti-biofilm activity against multiple human

pathogens. Although this work is limited to one Dictyostelid species, it is likely that many Dictyostelids harbor similar abilities to disrupt bacterial biofilms and represent a trove of potential candidates for use as therapeutics in treating bacterial infections exacerbated by biofilm formation. Further studies involving multiple Dictyostelid species' anti-biofilm products and bacterial species' biofilms are warranted to fully exploit the opportunities within this newly discovered phenomenon.

5.1.1 Contributions to Identification of Dictyostelid Mechanisms of Biofilm Dispersal

Results indicating that *S. epidermidis* OCT, presumably modified by Dictyostelid intervention, is partially or wholly responsible for anti-biofilm activity recoverable from *P. pallidum* predation of *S. epidermidis* was certainly unanticipated. This strategy may be more common than previously considered. Inevitably, prey bacteria must harbor mechanisms necessary to disperse cells from their biofilms to colonize new environments, and the biofilm EPS chemistries and means of dispersal are likely to be idiosyncratic. Adopting methods to generally co-opt multiple prey bacteria's dispersal mechanisms would represent a stunningly advantageous adaptation by Dictyostelids that allow their polyphagous existence in soil. Unfortunately, the putative protein or small molecule originally produced by *P. pallidum* to accomplish this activity has yet to be identified. However, this evidence contributes a more comprehensive understanding of Dictyostelid predation mechanisms that can be modelled for anticipated studies regarding biofilm dispersion in this field.

Identification of bacterial OCT as associated with biofilm dispersal may also contribute to understanding of *S. epidermidis* physiology and a nascent understanding of potential new functions of the OCT protein beyond nitrogen metabolism. It is also possible that OCT is employed in other bacterial species' biofilms for similar purposes and that Dictyostelid-modified *S. epidermidis* OCT is also capable of biofilm dispersion in these cases. Assuming that OCT is

conclusively recognized as being responsible for biofilm dispersion, the exact means by which it accomplishes dispersion is also yet to be understood. Beenken *et al.* explain that, under anaerobic conditions not unlike those found in the center of a biofilm, bacteria are capable of ATP generation via the catabolism of arginine involving catabolic OCT as part of the arginine deiminase pathway.[9] Given that catabolic OCT is upregulated in *Staphylococcus* biofilms compared to cells from exponential-phase growth,[9] Dictyostelids may be interfering with *S. epidermidis* anaerobic metabolism which forces the cells to release themselves from low oxygen tension conditions found within the biofilm after a resumption of aerobic metabolism. Additionally, pH perturbation via inhibition of ammonia production from the arginine deiminase pathway used to balance environmental pH[9] may coerce bacteria to degrade biofilm EPS to escape a harsh local environment.

5.2 Recommendations for Future Work

5.2.1 Use of In Vivo Wound and Device Infection Models

Given the involvement of host immune system and host proteins, such as those found in serum and the extracellular matrix, and their exploitation by bacterial surface extracellular matrix binding proteins, efficacy investigations of potentially therapeutic anti-biofilm compounds *in vitro* is severely limited. Animal models of implanted device infections or wound infections would address these limitations. Bona fide wound models incorporate the aerobic and anaerobic environments of the wound surface and interior. Additionally, the use of porcine models would be particularly convenient due to their suitability for investigation of wound healing. Despite higher costs, porcine wound models are recognized as superior to murine wound models due to the contraction of the panniculus carnosus and its involvement in murine wound healing.[10] Therefore, establishment of biofilm infections in a porcine wound model and subsequent treatment with D-IABC would provide stronger evidence of therapeutic potential.

5.2.2 Genetic Knockout and Neutralizing Antibody Studies

A main limitation of the work presented here is the lack of conclusive identification of the Dictyostelid genes responsible for anti-biofilm activity. Knockout screening may identify *P. pallidum* genes responsible for anti-biofilm activity. Although indiscriminate genetic screening may be ultimately required, several known proteins could be targeted for knockout studies that have already been identified as being secreted by Dictyostelids during development.[11] Alternatively, neutralizing antibodies against catabolic OCT could be used to prevent anti-biofilm activity even in unpurified D-IABC, indicating the necessity of its involvement without removing cooperative proteins or small molecules that potentially assist in its function.

5.2.3 Expansion of Target Biofilms to ESKAPE Pathogens and Other Species

Recent studies by collaborators[8] have indicated that Dictyostelids possess the ability to disrupt biofilms of multiple bacterial species, including pathogenic species of bacteria. Expanding the scope of studies to additional bacterial species would represent a natural extrapolation of investigations presented here. Medically relevant species whose infections involve biofilm formation such as members of the ESKAPE pathogens would serve as appropriate targets for future work. With the advent of additional bacterial species in these studies, multispecies biofilms could also be used in anti-biofilm assays given that multispecies biofilms possess greater medical relevance.

5.3 References

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