Social dynamics & adaptive strategies of microbes

URVISH TRIVEDI
PhD Thesis
Department of Biology
Section of Microbiology
Social dynamics & adaptive strategies of microbes

PhD Thesis by
Urvish Trivedi

Academic Advisor
Prof. Søren J. Sørensen

Department of Biology, Section of Microbiology
University of Copenhagen
Cover Page
Confocal laser scanning micrograph depicting a section of coagulated wound-like media containing *Staphylococcus aureus*.

Back cover
Confocal laser scanning micrograph depicting a section of coagulated wound-like media containing *Staphylococcus aureus*. 
Acknowledgements

This thesis reflects the efforts of a great many people that have paved the way for me to embark on a personal three-year journey filled with scientific discovery, excitement, and (to-be-expected) struggle. I consider myself truly fortunate in this aspect.

First and foremost, I would like to express my gratitude to my primary academic advisor Søren Sørensen for having given me the opportunity to undertake this project. Søren, day in and day out, you have been unknowingly setting an example for me with your dedication and humility towards your responsibilities as head-of-section. You have given me the freedom to pursue my own interests while providing me with the necessary guidance that has kept me on track. Your understanding nature and scientific encouragement have been invaluable. I would also like to thank my co-advisor Mette Burmølle, who has always been extremely understanding and encouraging. Mette, you have supported me a countless number of times during meetings and conferences in my many attempts to engage constructive debates. I could not have asked for better advisors.

Kendra Rumbaugh, words cannot truly describe the level of appreciation and respect I have for you. I still remember the day I walked into your office for my HHMI scholar interview. Since then, you have continued to be my scientific mentor and have always encouraged me to strive for the better. I think it is safe to say that without your tutelage, I would not be where I am today. I am forever indebted.

I would like to fire off a special thanks to the group in Texas. Cody Fell, I thank you for the tremendous amount of help in getting this project to the finish line. You have put in an immense amount of time and hard work purely out of scientific curiosity. Jake Everett, your insights into imaging have ignited a newfound passion of confocal microscopy and histopathology within me. Rebecca Gabrilaska and Angel Cueva, I would also like to thank you for your help during the many exhausting studies I was running during my stay. Derek Fleming and Hannah Zhao, I would like to thank you for graciously hosting me and providing me with the great company for the entirety of my internship. All of you are great professional colleagues and friends.

At the Section of Microbiology, I would like to thank Jonas Madsen for his collaborative efforts and helpful discussions for the many strange questions regarding evolution biology, method optimizations, and graphics. I also thank Anette Loth and Karin Vesteberg who have been very helpful in the laboratory and have helped me with last minute experiments more times than I can count. Never-to-be-forgotten are the members of the biofilm group, both the alumni and present members alike, who have always engaged in any discussion brought forth purely for the sake of expanding knowledge. Amongst those ranks, also stand my office mates (Jakob H., Henriette, Prem, Wenzheng, Nanna, Jakob R., Xuanji) and the ones in the lunch club (Sam, Leise, Stefan, Tue, Lars). It has been a pleasure being a part of the MME family!

Finally, I would like to thank my family and friends for their love and support. They have made a great deal of self-sacrifice for the betterment of my education and personal journey here in Denmark. To them, I owe my deepest gratitude.

Urvish Trivedi, Copenhagen 2017
Summary

In life, organisms seldom exist in isolation as autonomous units, but rather as social individuals that are shaped by interactions with their local environment. The emergence of social evolution theories has been a game changer for the field of microbiology. We now know that bacteria, like many other gregarious organisms, display a wide array of social traits. In this sense, social evolution theories have been indispensable for understanding how bacteria function as multicellular communities where both coordinated and uncoordinated group behaviors can transpire that ultimately affect their fitness. Recently, the significance of these bacterial social interactions during infections has come to light, spurring a new wave of questions and research interests within microbiology. However, in understanding these complex interactions, a majority of the studies fail to recreate the environment encountered by bacteria in clinical settings. Therefore, we placed a particular emphasis on using experimental models that reflect physiological conditions. This thesis is a marked departure from and yet a natural extension of social evolution research and clinical sciences. It bridges the two fields by studying microbial interactions in conjunction with a medical approach, a combination that is rarely explored experimentally but provides a novel and detailed understanding of why pathogens behave the way that they do.

In the introductory chapters, the most important concepts of hemostasis are introduced and hereafter used to describe the unique virulence factors of *Staphylococcus aureus*, their infection-specific roles, and the potential social nature of their expression in mixed communities. Thereafter, the focus shifts onto *Pseudomonas aeruginosa*, its adaptive strategies, and its secondary messenger c-di-GMP. Both chapters conclude with a brief discussion regarding potential future directions of research. This PhD-thesis has resulted in one published manuscript in a peer-reviewed journal and the production of four draft manuscripts. Manuscripts 1–4 are the product of a logical streamlined effort to describe the role of *S. aureus*-induced clotting during infections in a social evolution context. Manuscript 5 focuses on a specific adaptive strategy utilized by *P. aeruginosa* in coping with environmental changes.

Manuscript 1 is a critical discussion on the conflicting opinions about biofilms in infections. The main target of this review is the initial assessment and treatment methods employed by hospital clinics for treating chronic wounds. It uses a combination of scientific approach with clinical reality to separate fact from fiction in order to provide clear recommendations for understanding, diagnosing and treating biofilm-related infections. We address some of the fundamental issues overlooked by clinicians and provide a possible solution for improving the future direction of therapy plans – a clinically relevant *in vitro* model, WLM.

The significance of the biofilm phenomenon was extended in Manuscript 2, where *ex vivo* samples of human blood and the WLM was used in combination with advanced imaging to demonstrate the relative abundance of physiological components and the contribution of coagulases towards a host-derived matrix. In addition to this, we address the pressing question of whether or not the biofilm matrix itself is a shared resource, bringing forth a discussion that both challenges and expands the constitution of infectious biofilms; all the while providing the
first social evolution perspective on *S. aureus*-induced clotting of blood and plasma. This work also provides an inexpensive *in vitro* clinical model that can be employed in a variety of future studies dealing with infectious pathogens.

The realization that coagulases are able to function as public goods prompted us to ascertain whether or not this was true for *in vivo* infections involving mixed communities. In **Manuscript 3**, we established the virulence consequences and mixed community benefits associated with coagulases during the hematogenous spread of *S. aureus* in a murine abscess model. This is the first study to define coagulases as public goods where the defining pathophysiological events of staphylococcal abscesses were taken into account. A key finding of this study was the depth of clinical significance in relation to mixed communities being able to access the aforementioned public goods.

The previous findings were established for mixed communities of *S. aureus* and isogenic variants. However, a vast majority of chronic infections are known to harbor more than one type of strain. The most common occurrence of which are *S. aureus* and *P. aeruginosa* coinfections. In **Manuscript 4**, we established the contribution of coagulases towards a polymicrobial community. In addition to the methods and models employed previously, we used a murine chronic infection model. This study demonstrates that the ability of coagulases to serve as public goods was not limited to mono-species infections, but also applied to polymicrobial infections harboring gram-negative strains such as *P. aeruginosa*.

From here, we take a leap into a different topic for **Manuscript 5**, responsive phenotypic switching in *P. aeruginosa*. This study examined how environmental changes influence the population dynamics and evolution of this opportunistic pathogen. In carrying out our long-term evolution experiments, we found that specialists did occur for each of the opposing phenotypes, but generalists also emerged within the population. Although the specialists were able to outcompete the generalists in conditions favoring their respective phenotype, we found that the generalists were favored when upon relocation to conditions favoring opposing phenotypes. We found that the major fitness determinant for transitioning between opposing phenotypes was the optimization for regulating intracellular levels of c-di-GMP.
Resume'

Levende organismer forekommer sjældent alene som autonome enheder men snarere som sociale individer, der er præget af interaktioner i deres lokale miljø. Udviklingen af sociale evolutionsteorier har været en 'game changer' for mikrobiologien. Vi ved nu, at bakterier, som mange andre gruppeorganismer, har en bred vifte af sociale træk. I denne sammenhæng har sociale evolutionsteorier været uundværlige for at forstå, hvordan bakterier fungerer som multicellulære samfund, hvor både koordineret og ukoordineret gruppeadfærd kan forekomme, og det vil i sidste ende påvirke deres fitness. For nylig er betydningen af disse sociale interaktioner med hensyn til infektioner blevet belyst, idet der spores en ny bølge af spørgsmål og forskningsinteresser inden for mikrobiologi. De fleste undersøgelser af disse komplekse interaktioner undlader forsøg på at genskabe det miljø bakterier møder i kliniske miljøer. I dette arbejde lægges særlig vægt på at bruge eksperimentelle modeller, der afspejler fysiologiske forhold. Denne ph. d. afhandling er derfor en markant afvigelse fra og dog en naturlig udvidelse af social evolutionsforskning og klinisk videnskab. Den bygger bro mellem de to felter ved at studere mikrobielle interaktioner i forbindelse med et medicinsk approach, en kombination der sjældent undersøges eksperimentelt, men giver en ny og detaljeret forståelse for, hvorfor patogene opfører sig som de gør.


Manuskript 1 er en kritisk diskussion om modstridende opfattelser af biofilms betydning i infektioner. Hovedformålet med dette review er at evaluere de indledende vurderings- og behandlingsmetoder, der anvendes af hospitalsklinikker til behandling af kroniske sår. Vi bruger en kombination af videnskabelig approach og klinisk virkelighed for at adskille fakta fra fiktion med det formål at give klare anbefalinger til forståelse, diagnosticering og behandling af biofilm relaterede infektioner. Vi behandler nogle af de grundlæggende problemer, overset af klinikere, og giver en mulig løsning til fremtidige terapiplaner - en klinisk relevant *in vitro*-model, WLM.

Forståelsen af biofilms betydning blev udvidet i Manuskript 2, hvor *ex vivo* prøver af humorant blod og WLM blev anvendt i kombination med avanceret billedbehandling ved at demonstrere den relative mængde af fysiologiske komponenter og koagulase bidrag til en værstspezifik matrix. Herudover præsenterer vi det presserende spørgsmål om, hvorvidt biofilmatrixen selv er en fælles ressource, og der afsluttes med en diskussion, der både udforder og udvider
opfattelse af infektiøse biofilm; på denne måde præsenterer vi for første gang et socialt evolutionsperspektiv på *S. aureus*-induceret koagulation af blod og plasma. Dette arbejde giver også en billig *in vitro* klinisk model, som kan anvendes i en række fremtidige undersøgelser, der beskæftiger sig med patogene bakterier.

Konstateringen af, at koagulaser kan fungere som ‘public goods’, fik os til at undersøge om dette var tilfældet eller ej for *in vivo*-infektioner med blandede samfund. I **Manuskript 3** fandt vi virulens konsekvenser og fordele ved blandede samfund associeret med koagulaser ved *S. aureus* hæmatogene spredning i en muse abscess-model. Dette er den første undersøgelse af at definere koagulaser som ‘public goods’ hvor de patofysiologiske mekanismer i stafylokok absesser blev taget i betragtning. Et nøgleresultat af denne undersøgelse var en dybere klinisk betydning med hensyn til at blandede samfund kunne opfattes som de tidligere nævnte ‘public goods’.

De tidligere resultater blev opnået med blandede samfund af *S. aureus* og isogene varianter. Imidlertid vides det at langt størstedelen af kroniske infektioner huser mere end en slags bakterier og de hyppigst forekommende bakterier er *S. aureus* og *P. aeruginosa*-koinfektioner. I **Manuskript 4** undersøgte vi koagulasers bidrag i et polymikrobielt samfund. Udover de tidligere anvendte metoder og modeller anvendte vi en kronisk muse infektionsmodel. Denne undersøgelse viser, at koagulasernes evne til at tjene som ‘public goods’ ikke var begrænset til enkelte arter, men også fungerer ved polymikrobielle infektioner, som blandt andet indeholder den gram-negative *P. aeruginosa*.

**Manuskript 5** handler om responsivt fænotypisk skift i *P. aeruginosa*. I dette arbejde undersøgte vi, hvordan miljømæssige ændringer påvirker populations dynamik og udvikling af denne opportunistiske patogen. Vores langsigtede evolutions eksperimenter viste, at specialister forekom for hver af de forskellige fænotyper, men generalister opstod også inden for populationen. Selv om specialisterne kunne udkonkurrere generalisterne under forhold, der favoriserede deres respektive fænotype, fandt vi ud af, at generalisterne blev favoriseret efter flytningen til forhold der fremmede den modsatte fænotype. Vi fandt ud af, at den vigtigste fitness determinant for skiftet mellem modstående fænotyper var optimering for at regulere intracellulære niveauer af c-di-GMP.
Table of Contents

List of Manuscripts

Introduction

Chapter I – Social evolution of coagulases
  Primary hemostasis 3
  Secondary hemostasis 3
  Fibrinogen & Fibrin 4
  Staphylococcus aureus & The ECM 7
  The hemostasis factors of S. aureus 10
  Social evolution 12
  The biofilm phenotype 19
  Septic thrombosis 20
  Antimicrobial tolerance 23
  Complement & Phagocytes 23
  Abscesses 28
  Chronic wounds 31
  Secondary infections & Splenic tissue 33
  Conclusion & Future directions 35

Chapter II – Coping with changes
  Phenotypic plasticity & Genetic assimilation 39
  Bet-hedging 41
  The secondary messenger c-di-GMP 41
  Conclusion & Future directions 44

Bibliography

Manuscripts
  Manuscript 1 79
  Manuscript 2 95
  Manuscript 3 117
  Manuscript 4 141
  Manuscript 5 163

Appendix

Co-authorship Statements
List of Manuscripts

   Manuscript 1

   Manuscript 2

   Manuscript 3

   Manuscript 4

   Manuscript 5

The manuscripts to which I have contributed during my PhD but are not included in the thesis:


Xuanji Li, Urvish Trivedi, Asker Daniel Brejnrod, Martin Steen Mortensen, Søren Johannes Sørensen. Characterization of the Microbiome from Captive hamadryas baboon Reveals Specific Microbial Communities.
Foreword

This thesis will commence briefly. The introductory subchapters are intended to first provide the readers with the necessary details on their respective topic, followed by an open in-depth discussion on how these topics relate to the work of this thesis. In doing so, this thesis provides a comprehensive and critical review of itself. I do not provide a detailed discussion on the topics of polymicrobial infections, current pitfalls in the clinical lab rubric, and the ‘wound-like’ media in the introductory chapters because they are discussed in the critical review that is Manuscript 1.
Introduction
CHAPTER I – SOCIAL EVOLUTION OF COAGULASES

The aim of this chapter is to lay a foundation of the basic concepts of hemostasis, social evolution theories, and clinical biofilms. A grasp of this knowledge will enable the readers to better understand and appreciate the significance of staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp), the two fascinating clotting factors of *Staphylococcus aureus*. Subsequently, these inaugural concepts will extend into the topic of pathophysiology (mainly immunology) by highlighting the social interactions associated with Coa and vWbp during specific types of infections.

Since the formal characterization of *S. aureus* in 1884, there has been an increasing interest in the ability of this opportunistic pathogen to interact with the physiological components of its hosts. The significance of these characteristics has, in part, been made possible by the mounting number of studies analyzing the role of various *S. aureus* virulence factors during infection. Here we provide an overview of the virulence factors that could potentially play a role in the infection models employed during this thesis. Our current knowledge on the social interactions regarding a majority of virulence factors is lacking; but here we systematically address the benefits of Coa and vWbp in mixed communities by analyzing their contribution towards antimicrobial tolerance, survival/persistence, and progression of disease. We conclude the chapter with a brief summary of our findings and envision future research directions.
Hemostasis, defined as ‘the arrest of bleeding’, is derived from the Ancient Greek roots *haima* meaning blood, and *stasis* meaning to stop. Primary hemostasis is the formation of a ‘platelet plug’ in response to small injuries in microvessels in mucosal tissue (e.g. respiratory, gastrointestinal, and genitourinary tracts). Endothelial injury exposes subendothelial extracellular matrix (ECM) proteins (e.g. collagen, laminin, and fibronectin) and von Willebrand factor (vWF) that bind to and promote adhesion of circulating platelets [1]. Once the platelets adhere, they become activated and recruit additional platelets to the site of injury, and fibrinogen forms bridges between activated platelets to form the platelet plug. The negatively charged phospholipid (phosphatidylserine, PS)-bearing cell membranes are usually provided by activated platelets, but leukocytes and erythrocytes, which are incorporated into the clot, can also express PS on their cell surfaces [2]. Negatively charged membranes play a key role for the assembly of coagulation factors, whose significance will be further highlighted in the following sections. Normally the endothelium is a physical barrier separating circulating platelets from thrombogenic substances (materials that promote clotting) such as ECM proteins present in the extravascular space. This allows thrombogenic substances to remain passive until some stimulus converts them to their active form. Depending upon the location and degree of injury, secondary hemostasis (coagulation) is initiated simultaneously with primary hemostasis where fibrin further stabilizes the platelet plug. For simplicity, we will refer to secondary hemostasis as coagulation from here on out.

**SECONDARY HEMOSTASIS**

Coagulation of blood or extracellular fluids is a highly sophisticated physiological mechanism in response to injury, particularly in larger blood vessels where the platelet plug alone is insufficient to stop the hemorrhage. Physiological coagulation is divided into three distinct pathways: extrinsic, intrinsic, and common. Under physiological conditions, the pathways are controlled by a cascade of highly regulated serine proteases that limit blood loss and prevent systemic coagulation [3]. The constituents involved in these pathways consist of cells, enzymatic and non-enzymatic coagulation factors, protein substrates, calcium, and PS-bearing membranes. The negatively charged PS membranes act as a physical scaffold for coagulation and are essential for fibrin formation. They provide a binding site for the assembled coagulation factor complexes, enhancing their activity and protecting them from inhibitors. Coagulation factors are identified as Roman numerals, based on the order in which they were discovered historically (Table 1), e.g. fibrinogen is identified as coagulation factor I (fI). The zymogens and cofactors are further differentiated by their inactive and activated state, where the small ‘a’ after the factor number denotes the activated forms of the proteins, e.g. fXIII is the zymogen and fXIIIa is the active enzyme (Fig. 1 and SI.1). The extrinsic pathway is considered as the first step in plasma-mediated hemostasis and is activated by tissue factor (TF) expressed in the subendothelial tissue. Injury exposes TF, which binds plasma fVII and calcium to form the TF-fVIIa-Ca$^{2+}$ complex (extrinsic tenase) that converts FX to Fxa (Fig. 1 and SI.1) [4, 5]. Fxa then activates FV. After which, Fxa and FVa form the prothrombinase complex (fXa-FVa-fII) that cleaves prothrombin to thrombin (Fig. 1) [3]. The extrinsic tenase is inhibited by tissue factor pathway inhibitor (TFPI), resulting in only a small amount of thrombin being generated initially (Fig. SI.1). However, the already
generated thrombin then amplifies its own production on PS-rich surfaces by further activating fXI of the intrinsic pathway along with fV and fVIII, the intrinsic and common pathway cofactors [6]. The ultimate product of the intrinsic pathway is fIXa, which forms the fIXa-fVIIa-Ca²⁺ complex (intrinsic tenase) that amplifies the activation of fX. The subsequent prothrombinase complex then generates an explosive burst of thrombin from prothrombin. Thus, positive feedback loops from activated factors and the intrinsic pathway amplify the degree of clotting (Fig. 1) [3]. The coagulation cascade culminates in the conversion of circulating fibrinogen to insoluble fibrin by thrombin. In effect, coagulation is one of the most important physiological pathways.

Figure 1. The extrinsic and intrinsic coagulation pathways work in concert, which then converge into a single common pathway that is ultimately responsible for the production of thrombin, which catalyzes fibrinogen cleavage into fibrin. (Reproduced and adapted from the BSAVA Manual of Canine and Feline Clinical Pathology, 3rd edition.)

FIBRINOGEN & FIBRIN

Fibrinogen is an abundant plasma glycoprotein that is a central player in the coagulation cascade. Weighing in at 340-kDa, this soluble glycoprotein is present at concentrations of 2–4 mg/mL (6–12 µM) in blood [7]. Native fibrinogen is a hexameric protein (symmetric dimer of trimers) consisting of three pairs of nonidentical polypeptide chains: Aα, Bβ, and γ, which are linked together by disulfide bonds [8-10]. The six chains come together at their N-termini in the central E domain, whereas their C-termini extend outward to form globular domains (Fig. 2) [11, 12]. Thrombin cleaves the A and B peptide extensions from the N-termini of the α- and β-chains of fibrinogen, releasing them as A and B fibrinopeptides [13]. This initiates a structural
rearrangement between adjacent polypeptides, resulting in elongation and lateral aggregation of fibrin, followed by the fXIII mediated cross-linking of fibrin aggregates to form an insoluble polymer meshwork, known as a clot [12, 14]. To complete the healing process, the clot is ultimately broken down in a process called fibrinolysis by the activated form of plasminogen called plasmin – a broad-spectrum fibrinolytic protease.

<table>
<thead>
<tr>
<th>Coagulation Factors (Table 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor number</strong></td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>V</td>
</tr>
<tr>
<td>VI</td>
</tr>
<tr>
<td>VII</td>
</tr>
<tr>
<td>VIII</td>
</tr>
<tr>
<td>IX</td>
</tr>
<tr>
<td>X</td>
</tr>
<tr>
<td>XI</td>
</tr>
<tr>
<td>XII</td>
</tr>
<tr>
<td>XIII</td>
</tr>
</tbody>
</table>

Table 1. The clotting factors are numbered I through XIII according to the order of their discovery. *The liver requires the fat-soluble vitamin K to produce the factor.
Supplementary introduction figure 1. Physiological blood coagulation cascade. (Reproduced from https://www.qiagen.com/fi/shop/genes-and-pathways/pathway-details/?pwid=64) © 2009 QIAGEN, all rights reserved.
Staphylococcus aureus was first isolated from the pus of surgical wounds by Sir Alexander Ogston [15]. He initially referred to the sphere-shaped bacteria as ‘micrococci’, and later named the organisms staphylococci due to their characteristic appearance – grape-like clusters (staphyle in Greek). S. aureus is a gram-positive facultative anaerobe that is a common human commensal, colonizing the skin and nasopharynx. It is also a formidable opportunistic pathogen that faces modern medicine. S. aureus-related clinical complications range from localized soft tissue to invasive infections such as endocarditis, metastatic infections of joints, kidneys, and lungs with progression to sepsis [16]. With the widespread emergence of community-acquired methicillin-resistant S. aureus (CA-MRSA), which is also one of the leading causes of nosocomial infections, treatments using last-line antibiotics have become more challenging [17]. Its remarkable pathogenic potential and ability to thrive in a wide range of sites within the host is due to an array of its virulence factors, including adhesins, toxins, and immune evasion proteins [18]. However, an in-depth discussion on all of these topics may prove to be beyond the scope of this thesis. Therefore, we will focus on the interactions of S. aureus with the host extracellular matrix (ECM) components, and their significance during infection.

S. aureus has evolved a highly adaptive and versatile strategy to interact with host proteins such as collagen, laminin, vitronectin, fibronectin, fibrinogen, and fibrin. S. aureus is equipped with a large set of finely-tuned virulence-associated gene products known as adhesins/invasins that facilitate its interactions with the host ECM. The adhesin/invasin comprises a subgroup of cell wall anchored proteins, termed MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) and a subgroup of SERAMs (Secretable Expanded Repertoire Adhesive Molecules), which are released, but mainly surface-associated proteins [19, 20]. S. aureus produces a surprising number of cell wall-anchored and secreted proteins that bind fibrinogen. However, not all of these proteins appear to contribute appreciably to clotting, and will only be covered briefly. From the MSCRAMM subgroup, we include fibronectin binding proteins (FnbpA and FnbpB), and fibrinogen/fibrin binding proteins such as clumping factor A and B (ClfA and ClfB). From the SERAM subgroup, we include fibrinogen/fibronectin binding proteins such as the extracellular adherence and protein (Eap) and the extracellular matrix binding protein (Empbp).

Nearly a century ago, H. Much first reported the clumping of S. aureus in the presence of plasma [21]. Clumping was later found to be independent of coagulation and required the presence of fibrinogen [22]. S. aureus utilizes clumping factor A (ClfA) to interact with the extreme C-terminus of the γ-chain of fibrinogen (Fig. 2) [23-25]. ClfA adopts a three-step ‘dock, lock and latch’ mechanism to bind fibrinogen [26, 27], allowing the host protein to act as a bridge between neighboring cells (Fig. SI.2). In addition to fibrinogen, ClfA displays similar binding affinity for fibrin, allowing it to mediate clumping with both forms of the molecule [28, 29]. S. aureus expresses a second clumping factor, ClfB, that is structurally similar to ClfA [30]. Both interact with the distal ends of fibrinogen dimer, but unlike ClfA, ClfB binds to the α-chain (Fig. 2) [31]. In addition to fibrinogen, ClfB also binds cytokeratin 10 and loricrin, facilitating S. aureus adhesion to the nasal epithelium [32-34]. Almost all S. aureus clinical isolates express ClfA and ClfB, making clumping in the presence of plasma a nearly universal phenotype.
S. aureus produces two additional proteins that share structural homology to its clumping factors. As their name implies, the fibronectin binding proteins A and B (FnbpA and FnbpB) were both originally classified for binding fibronectin [35-37], a plasma glycoprotein that is less abundant than fibrinogen [38]. However, it was later discovered that FnbpA and FnbpB could also bind fibrinogen and elastin [39-42]. Although both proteins interact with the same region of fibrinogen as ClfA (Fig. 2) [43], neither FnbpA nor FnbpB seem to contribute towards clumping in plasma. This could be due to a steric exclusion of fibrinogen when FnbpA and FnbpB have already bound fibronectin [44].

Figure 2. Schematic of fibrinogen, showing where each of the discussed MSCRAMMs bind. Clumping factor A, ClfA; fibronectin binding protein A and B, FnbpA, and FnbpB bind to the γ-chain of fibrinogen. Clumping factor B, ClfB binds to the α-chain of fibrinogen. (Adapted from Hassouna, H. I. (2009). Thrombophilia and hypercoagulability. Medical Principles and Practice. International Journal of the Kuwait University, Health Sciences Center 18, 429–440.)

In addition to the MSCRAMMs discussed above, we considered two additional fibrinogen-binding proteins that belong to the SERAM subgroup. These include extracellular matrix and plasma binding protein (Empbp), and extracellular adherence protein (Eap). In general, less is known about the secreted fibrinogen-binding proteins, where both Empbp and Eap are thought to associate with the cell surface after secretion. Empbp (also known as Emp and Ssp) is known to interact with fibronectin, fibrinogen, and vitronectin [45]; however, the mechanism for binding these ligands has not yet been established. Eap (also known as Map) was originally characterized for its ability to bind fibrinogen, fibronectin, vitronectin, thrombospondin, and collagen [46, 47]. However, later work showed that Eap was not necessary for S. aureus binding to any of these ECM components, suggesting it might have alternative functions [48, 49]. At first glance, the binding of these proteins to similar ECM components may seem redundant. However, the maintenance of these genes with an apparent overlap in binding affinities suggests different roles for them in the complex pathogenic life cycle of S. aureus. The relevance of these gene products and their roles during infection will be further discussed in the coming sections. Fibrinogen is at the interface of host defense and pathogen virulence in S. aureus infection, therefore the significance of interacting with this host protein is apparent. Even though these
MSCRAMMs and SERAMs interact with fibrinogen, their binding to this glycoprotein does not precipitate fibrinogen cleavage and/or fibrin clot formation [Manuscript 2][45, 50].

The coordinated temporal expression of \textit{S. aureus} virulence factors is orchestrated by global regulatory loci such as \textit{Agr}, \textit{Sar}, \textit{Rot}, \textit{SigB}, and \textit{Sae}, which allow it to sense environmental changes and respond accordingly [51]. The \textit{saePQRS} system controls the expression of genes encoding several MSCRAMMs and SERAMs. In fact, with the exception of clumping factors, all of the genes discussed above are shown to be transcriptionally activated by the \textit{saeRS} two-component system [52]. Therefore, the \textit{saePQRS} mutant was analyzed for its ability to mediate clotting in plasma. Our phenotypic screens demonstrated that the \textit{saePQRS} mutant was unable to generate clots [Manuscript 2]. This was of interest because the \textit{sae} system also controls the expression of other secreted proteins belonging to the SERAM family such as the two prothrombin activating proteins, staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp).

In addition to limiting blood loss, coagulation is an ancient innate defense mechanism against invading pathogens that traps and immobilizes bacteria in a clot [53-55]. Much like phosphatidylserine (PS)-bearing membranes of host cells, bacteria are also present with negatively charged structures on their surfaces. The recognition of these structures results in a microbe-specific activation of the intrinsic pathway [56]. The auto-activation of fXII into fXIIa, subsequently converts prekallikrein and fXI into their active proteolytic forms, kallikrein, and fXla, respectively [57]. FXIa converges with the extrinsic pathway and ultimately leads to fibrin clot formation, while activated kallikrein cleaves the nonenzymatic cofactor kininogen and releases the proinflammatory peptide bradykinin, induces vasculature leakage and functions as a chemoattractant in recruiting additional immune cells [56]. However, the coagulation pathway is also the target of bacterial survival and immune evasive strategies. The ability of Staphylococcus aureus to clot plasma and blood was first reported in 1903 by Leo Loeb [58]. Since then, this trait has become the topic of interest for more than a century amongst both clinicians and microbiologists. S. aureus secretes two soluble coagulases that interact with the central coagulation zymogen, prothrombin, in a non-proteolytic manner to catalyze fibrinogen conversion to fibrin (Fig. 3).

Staphylococcal coagulase (Coa) and von Willebrand factor binding protein (vWbp) are the two hemostasis factors of S. aureus and the focus of Manuscripts 2, 3 and 4. Coa and vWbp are mosaic proteins that share secondary structural homology (25% corresponding amino acid identity) in their conserved D1 and D2 domains within the N-terminal half of the protein [59, 60]. The structure of the NH2-terminal half of Coa (residues 1-325) and vWbp (residue 1-263), is unique with a characteristic elbow-like fold between two domains (D1-D2) comprised of 3-helix bundles [59]. Structural and kinetic studies have demonstrated that both Coa and vWbp use a ‘molecular sexuality mechanism’ to activate prothrombin (ProT). Insertion of the first two NH2-terminal residues of Coa (Ile1-Val2) or vWbp (Val1-Val2) into the Ile16 pocket of ProT forms a salt bridge with Asp104, triggering a conformational change that induces a functionally active catalytic site in the zymogen [59, 60]. This interaction results in ProT•Coa or ProT•vWbp, tight stoichiometric complexes termed staphylothrombins that display a high binding affinity to fibrinogen. Staphylothrombins cleave the A and B fibrinopeptides of fibrinogen, thereby generating fibrin clots independent of the otherwise physiological regulatory steps of blood coagulation (Fig. 3). Despite the functional overlap of the two molecules, the C-terminal domains of Coa and vWbp are dissimilar [59, 61]. The C-terminal end of Coa is comprised of 2–8 tandem repeats of a conserved 27-amino-acid peptide [59], which allows Coa to associate with a molar excess of fibrinogen [62]. Whereas the C-terminal domain of vWbp includes a unique binding site for von Willebrand factor [61]. Regardless, both proteins allow S. aureus to usurp the coagulation cascade, without activation of the other clotting and inflammatory factors.

We analyzed the clotting ability of the community-acquired MRSA (USA300 LAC), and its isogenic mutants Δcoa that does not produce Coa; Δvwb that does not produce vWbp; and ΔcoaΔvwb that does not produce Coa and vWbp [Manuscripts 2, 3, and 4]. It should be noted that although both coagulases activate ProT and bind fibrinogen, vWbp binds both at a lower affinity than Coa [60, 61]. In the addition, the ProT•vWbp complex displays slower enzyme
kinetics than ProT*Coa, generating fibrin fibrils at a reduced rate [60]. This is in line with our observations, where the Δvwbp that still produces Coa was able to generate clots within the time frame allotted, but the Δcoa was not [Manuscripts 2 and 4]. Nevertheless, vWbp forms a complex with ProT, fibrinogen, and fXIII that is able to activate the transglutaminase activity of factor XIII [63]. This interaction further strengthens the fibrin clot with covalent crosslinks, where fXIII introduces ε-(γ-glutamyl)lysine cross-bridges between adjacent α- and γ-chains [63]. Therefore, since fXIII activity affects the 3-dimensional structure of the clot, we included the ΔcoaΔvwbp mutant, which was unable to generate clots [Manuscripts 2, 3, and 4].

**Figure 3.** Schematic of how *S. aureus* hijacks the common coagulation pathway that mediates fibrin clot formation. Coa, staphylocoagulase; vWbp, von Willebrand factor binding protein; and Sak, staphylokinase are shown in blue. Inset shows a representation of fibrinogen; arrowheads point to the thrombin cleavage sites. (Reproduced from Crosby, H.A. et al. (2016). *S. aureus* Aggregation and Coagulation Mechanisms. *Advances in Applied Microbiology* 96, 1–41.)

*S. aureus* can eventually break down this clot by secretion of staphylokinase (Sak) (Fig. 3). Similar to coagulation, physiological fibrinolysis can be viewed as a cascade of protease activation and inhibitory events part of the wound healing process. Sak binding of plasminogen induces a catalytically active site in the circulating blood zymogen. Normally the activity of plasmin is regulated by the endogenous inhibitor α-2-antiplasmin; however, the activity of the Sak-plasmin complex remains unaffected upon its binding to fibrin [64]. Therefore, by secreting Sak, *S. aureus* is able to unravel the fibrin meshwork, allowing individual cocci to disseminate away from the original clots onto new uninfected sites. So how does this fascinating trait relate to social interactions in microbial communities?
Social evolution is the study of how certain behaviors evolve over time. Social behaviors can be classified into four major groups: mutualism, selfishness, altruism, and spite [65, 66]. The different perspectives of social behaviors correspond to a common theme – the fitness consequences they entail for the actor and recipient (Fig. 4). Fitness is based on the total lifetime of the individual and its reproductive success. A behavior that increases the direct fitness of the actor and also benefits the recipient is considered mutually beneficial, whereas selfish behavior confers a benefit onto the actor but decreases the fitness of the recipient. A behavior that reduces the fitness of the actor but benefits the recipient is considered altruistic, whereas spiteful behavior results in a loss for both the actor and recipient. These behaviors are familiar patterns of sociality in metazoans such as insects, and vertebrates. However, recent technological advances have shown these theories to be highly relevant for microorganisms as well.

![Figure 4](image-url) Classes of social behaviors. The types of interactions are divided into four groups based on whether they are beneficial or costly to the actor and/or recipient.

The realization that bacteria participate in a number of social behaviors now supersedes the notion of what were once thought of as autonomous units. Microorganisms are particularly important in addressing social evolution theories because they allow for genetic manipulation, rapid generation times, and evolution experiments with a high level of control. In overturning our previous assumptions, we now know that bacteria can communicate and cooperate to perform activities such as dispersal, foraging, construction of microscopic cities, reproduction, chemical warfare and signaling [67-77]. Ideas combining both mechanistic and evolutionary approaches bear huge potential in understanding cooperative behaviors and their evolution. However, cooperation is a difficult behavior to explain – why should an individual carry out a costly behavior for the benefit of other individuals or the local group [65, 66, 78]? Charles Darwin himself alluded to this problem, noting how seemingly selfless workers in social insect colonies forgo reproduction for the benefit of the hive queen and the mutualism that occurs between nectar producing orchid and its insect pollinators. The main pillar for Darwin’s early arguments for the theory of evolution was competition – behavior most commonly associated with the idea of ‘survival of the fittest’. Understandably, Darwin himself was skeptical in how natural selection could favor cooperation:
“If it could be proved that any part of the structure of any one species had been formed for the exclusive good of another species, it would annihilate my theory, for such could not have been produced through natural selection.” (Darwin, 1859)

The fact that cooperation provides a benefit at the population or species level is not enough for it to be favored in the face of competition against selfish strategies. This dilemma is well known in the fields of economics and human morality where it is termed ‘the tragedy of the commons’. The tragedy is that as a group, individuals stand to benefit from cooperation, but cooperation is not stable because each individual can gain by selfishly pursuing their own short-term interests. Garrett Hardin showed this by means of an analogy: imagine a number of shepherds, each deciding how many sheep to graze on a shared pasture. Each shepherd stands to benefit by introducing additional sheep into the flock, even if it causes overgrazing. This is because our focal shepherd gains all the benefit, but pays only a fraction of the cost of overgrazing, which is shared between all the shepherds. Consequently, the individual shepherd stands to gain more than to lose from adding extra sheep. There are other numerous examples, such as fisheries and public vaccination programs, whereby anyone with access to a shared valuable resource has an interest in over-exploiting it, and it is in nobody’s interest alone to maintain it. We explore this dilemma through evolution experiments in Manuscripts 2 and 4, which demonstrate how the cooperative production of coagulases results in more robust clots and confers greater benefits as opposed to when the population is invaded by selfish individuals.

In microbiology, this dilemma regarding the evolution of cooperation is that of ‘public goods and cheaters’. Public goods are products manufactured by an individual that can be utilized by the producer or its neighbors (Fig. 5). For bacteria, these can be the numerous factors produced and released into the environment outside the cell membrane. We regard coagulases as our public goods of interest within the scope of our study [Manuscripts 2, 3, and 4]. Public goods lead to a dilemma because they are valuable resources that benefit all cells in a local group or population but are metabolically costly to produce [79]. Cheats are uncooperative, relatively selfish cells that can benefit from the public goods but do not produce them. Cheats can arise through mutation or migration, and in the absence of any mechanism to enforce cooperation, the cheats benefit from the cooperative behavior of its social partners without paying any of the costs. Therefore, by means of exploitation, cheats can gain a competitive advantage, outcompete the unconditional cooperators and overtake the population, eventually resulting in a tragedy of the commons. We found the cooperative expression of coagulases to be highly beneficial for the overall population during infection [Manuscripts 2, 3, and 4], whereas over-exploitation of these public goods led to a decline in population fitness [Manuscript 2 and 4]. This leads to the fundamental question of what makes cooperation evolutionary stable in the face of competition?

It wasn’t until a hundred after On the Origin of Species was published, that an evolutionary biologist – William Hamilton – provided the first full theoretical explanation that rationalized cooperation [65, 66, 78]. His work extended on Darwin’s original insight and made fundamental advances in our understanding of natural selection. Since then, a large amount of literature has been generated on the subject [73, 79-81], which divide possible explanations for cooperation into two broad groups: 1) Mutually beneficial cooperation – provides a direct fitness benefit to the actor that performs the behavior and outweighs the cost of performing the behavior.
2) Altruistic cooperation – decreases the direct fitness of the actor that performs the behavior. Mutualism may be selected for in situations where the individuals involved have a shared interest in cooperating, and where the advantage of cheating is removed or cooperation is enforced. Whereas altruism requires that cooperation is directed towards individuals who share cooperative genes. The crux of Hamilton’s argument was that the evolution of cooperation and altruism is dependent on genetic relatedness (kinship) between individuals [65, 66, 78], where the behavior is directed towards a family member or an individual that shares alleles at the variable genetic loci with the actor. This is termed ‘kin selection’ (also known as inclusive fitness theory).

“In brief outline, the theory points out that for a gene to receive positive selection it is not necessarily enough that it should increase the fitness of its bearer above the average if this tends to be done at the heavy expense of related individuals, because relatives, on account of their common ancestry, tend to carry replicas of the same gene; and conversely that a gene may receive positive selection even though disadvantageous to its bearers if it causes them to confer sufficiently large advantages to relatives.” (Hamilton, 1964)

By aiding a relative, the actor is still passing copies of its genes on to the next generation, hence the term ‘indirect fitness’. Hamilton’s rule, $rb – c > 0$, demonstrates when altruism is favored. The rule illustrates that cooperation is more likely to occur when $r$ or $b$ is high and $c$ is low, where $c$ is the fitness cost to the actor (altruist), $b$ is the fitness benefit to the recipient (beneficiary), and $r$ is their genetic relatedness. Given that kin selection requires a high degree of relatedness between cooperating individuals, how does one ensure a higher proportion of relatives in social groups than nonkin? Hamilton suggested two possible explanations for this: 1) Population viscosity (limited dispersal), and 2) Kin discrimination.

1) Population viscosity – keeps relatives together. In doing so, based on probability, the indiscriminate acts of altruism (such as sharing public goods) directed towards neighbors will have a higher chance of benefiting relatives, making them favorable. In this sense, communities
initiated by one or a small number of clonal lineages can be highly conducive to the evolution of cooperation. Population viscosity could potentially play a role in *Staphylococcus aureus*-induced coagulation, where the secreted coagulases and the fibrin structures primarily benefit the immediate neighboring cells [Manuscripts 2 and 3].

2) Kin discrimination – requires that an individual be able to distinguish relatives from non-relatives so as to preferentially aid its kin [65, 66]. One way of achieving this is through specificity, where the actor produces highly specific public goods that only its kin can utilize, thereby excluding non-relatives. The other involves reducing competition for closer relatives by directing harmful or spiteful behavior towards non-relatives. This can be achieved through ‘chemical warfare’ where toxins target non-relatives who cannot produce the necessary antidote. An extreme form of kin discrimination involves directing cooperative behavior at other individuals that carry ‘green beard’ genes. This gene causes a distinguishable phenotypic difference in the carrier (e.g. having a green beard) that can be recognized by the other individuals bearing the same gene who can adjust their behavior accordingly. However, a population of green beards is not immune to invasion and exploitation, because cheats can express a green beard without performing the cooperative behavior (a form of deception). Policing and punishment can also favor cooperation by reducing the fitness gains of cheating, which reduces the cost of cooperating. This behavior can result in either direct or indirect fitness benefits. For example, reducing the fitness of non-relatives who are competing with relatives frees up available resources for kin. This is termed enforcement (repression of competition).

The social evolution of cooperative behaviors depends on the degree of relatedness. Although the term genetic relatedness sounds familiar in a colloquial sense, from a social evolution perspective it is not so intuitive. The manner in which genetic relatedness is calculated between two individuals depends largely on the problem at hand. Manuscripts 2, 3, and 4 spotlight the consequences of variation for one single trait: whether or not the *S. aureus* strains generate clots. With respect to that, relatedness is measured at the locus or loci that control the behavior in question because it is the change in frequency of alleles at these loci that govern the evolution of the trait [82]. A null mutation that eliminates secretion of coagulases in *S. aureus* can produce a cheating strain that exploits the ones that continue to produce coagulases. This distinction is particularly important because the initial clonal population is subject to invasion and exploitation by spontaneous mutants that no longer share the interests in producing coagulases. Therefore, in our experiments, with all other parts of the genome being identical, *coa* and *vwbp* are the two genes we deem to be the fitness determinants. To avoid confusion, we further clarify the usage of terms where we regard Δ*coa* and Δ*coaΔvwbp* to be cheats and the wild-type LAC to be producers (cooperators) [Manuscripts 2, 3 and 4], whereas *Pseudomonas aeruginosa* is simply identified by its name [Manuscript 4]. First off, the genome of *P. aeruginosa* is distant to that of *S. aureus*, second, just because there is a significant niche overlap between the two strains during infection, *S. aureus* should not be expected to be altruistic towards *P. aeruginosa* because the two are not related to each other. Furthermore, if the trait in question is coagulation, then perhaps *P. aeruginosa* cannot be considered a cheat because it never possessed the necessary genes to produce coagulases or fulfill the requirements for participating in act of clotting in the first place.
In the most parsimonious sense, cheats require a mechanism for cheating others, where they intercept a trait meant for someone else or manipulate others to direct cooperation towards them. One of the ways of achieving the latter is through deception via signaling systems, which in bacteria is associated with quorum sensing (QS). This form of cell-to-cell communication employs small diffusible molecules known as autoinducers that function as signals to stimulate a concerted response and coordinate a number of bacterial behaviors. There are a series of fundamental steps involved in all QS systems: 1) Firstly, the autoinducers are synthesized intracellularly, which are then 2) actively or passively made available outside of the cells. 3) As the number of cells in a population continues to rise, so does the concentration of autoinducers, which causes 4) the autoinducers to accumulate above the minimal threshold for detection, at which point, cognate receptors bind the autoinducers and trigger signal transduction cascades that result in population-wide changes in gene expression [83]. It is through these steps that QS systems allow bacteria to synchronously control gene expression in response to changes in cell density and species complexity (e.g. kin v. nonkin).

Over the past couple of decades, many different QS systems have been recognized and shown to be unique for different groups of bacteria. For example, gram-negatives use acylated homoserine lactones (AHLs), whereas gram-positives use smaller, modified oligopeptides. Although these group-specific AHLs and oligopeptides facilitate intraspecies communication between the sender and receiver of the signal, interspecies signals such as autoinducer-2 (AI-2) can also facilitate cross-talk among different groups of bacteria. However, this form of communication can potentially leave the signaling channels open to cheating by individuals of the same species or individuals of another species that are able to ‘listen in’ on the group behavior but do not take part. Deceitful individuals can produce false signals or mimic response patterns to elicit a specific reaction or trait from cooperating individuals to gain a benefit. However, the off chance that \textit{P. aeruginosa} uses deceptive strategies to elicit the production of coagulases in our study is highly unlikely. Simply because this implies a cognitive intent from \textit{P. aeruginosa} to intentionally coerce \textit{S. aureus} into producing coagulases and generating clots, a trait with which, to our knowledge, it has no association whatsoever. It remains to be seen whether or not interspecies signals can stimulate \textit{S. aureus}-induced coagulation.

In making this distinction, one must consider that not all cheats need use deceptive methods to benefit from a specific trait. For example, \textit{P. aeruginosa} LasR mutant strains do not respond to quorum sensing signals but are able to exploit the cooperative public goods produced by other strains in response to quorum sensing [84-88]. However, one should bear in mind that in this case, the trait in question is LasR-mediated quorum sensing; a behavior for which the mutant strains did once possess the ability to participate in, thereby making them cheats that were cooperators in their evolutionary pasts. Therefore, with respect to that, one must recall the previous discussion where we clearly state that this is not the case for coagulases and \textit{P. aeruginosa}. However, one could argue that cheats could have not evolved from cooperators. For example, cuckoos (\textit{Cuculus canorus}) lay their eggs in warbler’s nests, exploiting the warbler’s parent-offspring interaction [89, 90]. Although the cuckoo is an obligate brood parasite, its selfish behavior should not be characterized to be strictly parasitic. This scenario can also be a form of cheating because it involves exploiting the cooperative behavior of others, which could potentially lead to a breakdown in cooperation. However, this form of cheating takes us back to
the dilemma of whether or not deception is involved. In making this distinction, perhaps a simpler explanation is that not every individual that benefits from a cooperative act is a cheat. Thus the benefits conferred onto *P. aeruginosa* from *S. aureus*-induced coagulation could simply be a form of bystander effect [Manuscript 4].

So far we have considered complex social behaviors that can be mediated through well-characterized molecular systems such as quorum sensing. Although whole-group coordination and cooperation can occur via communication, collective behavior patterns can also emerge independent of such systems. For example, consider schools of fish that exhibit a wide range of group structures that rapidly change in response to predators or prey items. Studies have demonstrated that such collective behaviors do not always involve active communication and coordination among constituent members as long as each individual upholds few individual responses: 1) move away from neighbors that are too close; 2) do not separate from the group; and 3) avoid predators [91-93]. Although this does not directly translate to microbes, it is an interesting point to consider for a predominant lifestyle state adopted by bacteria — biofilms, which is a prime niche for a number of behaviors that confer benefits onto individual cells. As a whole group, bacteria residing in biofilms can regulate a number of cooperative behaviors by QS systems that involve communication and coordination. Alternatively, one could argue that the individual cells within a biofilm avoid separation from the group because membership therein provides a range of direct benefits such as resource capture or digestive capacities to protection against a wide range of environmental challenges such as UV exposure, desiccation, predatory grazing, host immune cells, and antimicrobials [94, 95]. Under the influence of simple physical forces, heterogeneity in community physiology and spatial structure can emerge from the uncoordinated behavior of constituent members and add to the integrity of the biofilm as a whole. While the ubiquity of bacteria has become all the more familiar, the dissection of the many layers of bacterial interactions and the social evolution theories that shape them are only just now coming to light.
Glossary.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actor</td>
<td>the focal individual performing a behavior.</td>
</tr>
<tr>
<td>Altruism</td>
<td>a behavior that increases another individual's fitness at a cost to one’s own.</td>
</tr>
<tr>
<td>Cheat</td>
<td>an individual who does not cooperate (or cooperates less that their fair share), but can potentially gain the benefit from others cooperating.</td>
</tr>
<tr>
<td>Cooperation</td>
<td>a behavior that benefits another individual (recipient), and which is maintained (at least partially) because of its beneficial effect for the recipient.</td>
</tr>
<tr>
<td>Cue</td>
<td>something that can be used by an individual as a guide for future action.</td>
</tr>
<tr>
<td>Deception (manipulation)</td>
<td>a behavior that alters the behavior of another individual, usually to the benefit of the actor and to the cost of the other individual.</td>
</tr>
<tr>
<td>Direct fitness</td>
<td>the component of fitness gained through reproduction; the component of fitness due to one’s own behavior.</td>
</tr>
<tr>
<td>Enforcement (repression of competition)</td>
<td>when the selfish advantage of cheating is removed.</td>
</tr>
<tr>
<td>Green beard</td>
<td>a hypothetical gene that causes in carries both a phenotype that can be recognized by conspecifics (e.g. a ‘green beard’) and a cooperative behavior towards conspecifics who show a green beard.</td>
</tr>
<tr>
<td>Hamilton’s rule</td>
<td>a condition ((rb - c &gt; 0)) that predicts when a trait is favored by kin selection, where (c) is the cost to the actor performing the behavior, (b) is the benefit to the recipient who the behavior is directed towards, and (r) is the genetic relatedness between those individuals.</td>
</tr>
<tr>
<td>Inclusive fitness</td>
<td>“the effect of one individual’s actions on everybody’s numbers of offspring […] weighted by the relatedness; the sum of direct and indirect fitness gained from aiding related individuals.</td>
</tr>
<tr>
<td>Indirect fitness</td>
<td>the component of fitness gained from aiding the reproduction of non-descendant relatives.</td>
</tr>
<tr>
<td>Kin discrimination</td>
<td>when behaviors are directed towards individuals depending on their relatedness to the actor.</td>
</tr>
<tr>
<td>Kin selection</td>
<td>a process by which traits are favored because of their beneficial effects on the fitness of relatives.</td>
</tr>
<tr>
<td>Mutual benefit</td>
<td>a benefit to both the actor and the recipient.</td>
</tr>
<tr>
<td>Mutualism</td>
<td>two-way cooperation between species.</td>
</tr>
<tr>
<td>Public goods</td>
<td>a resource that is costly to produce, and provides a benefit to all the individuals in the local group or population.</td>
</tr>
<tr>
<td>Recipient</td>
<td>an individual who is affected by the behavior of the actor.</td>
</tr>
<tr>
<td>Relatedness</td>
<td>a measure of genetic similarity between two individuals, relative to the average.</td>
</tr>
<tr>
<td>Signal</td>
<td>something that alters the behavior of another individual, which evolved because of that effect, and which is effective because the receiver's response has also evolved.</td>
</tr>
<tr>
<td>Spiteful</td>
<td>a behavior that decreases another individual’s fitness at a cost to one’s own.</td>
</tr>
<tr>
<td>Tragedy of the commons</td>
<td>a situation when individuals would benefit from cooperating, but cooperation is unstable because each individual gains by selfishly pursuing their own short-term interests.</td>
</tr>
</tbody>
</table>
In 1842, Henry David Thoreau published his essay *Natural History of Massachusetts* in *The Dial*. In it he wrote:

“Nature will bear the closest inspection; she invites us to lay our eye level with her smallest leaf, and take an insect view of its plain.” (Thoreau, 1842)

Without a doubt, many scientists have found their inspiration from naturally occurring phenomenon, but one, in particular, peered into microscopic cities and pioneered a golden age of research within biology. During his climb of the Bugaboos in eastern British Columbia, Canada, Bill Costerton noticed that the rocks of the alpine stream were covered with a slippery ‘slime’. Together with his colleague Gill Geesey, Costerton performed microscopic analysis on the submerged rocks and confirmed that microorganisms attached readily to the surface of the rocks [96]. Later he would go on to publish his defining paper, *How Bacteria Stick* [97], which has spurred an entire generation of scientists into deciphering the predominant lifestyle of bacteria – biofilms.

Today, we know the occurrence of biofilms is widespread, ranging from the seabed to medical implants in patients. Traditionally, a biofilm has been defined as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” [98]. The biofilm mode of growth is usually depicted as a series of discrete stages in a life cycle based on experimental findings using primarily the flow cell biofilm setup (Fig. 6) [99]. Stage 1 is initial attachment, during which individual cells reversibly adhere to a surface [100]. Cells are still capable of independent movement during this noncommittal stage [101], and may actually detach from the surface to resume their planktonic lifestyle. Differentiation to biofilm formation results in the initiation of Stage 2, where cells irreversibly begin to exude exopolysaccharide and irreversibly fixate themselves onto the surface. The term ‘irreversible’ is used here to imply firm attachment and should not be confused to mean a loss of motility amongst the constituent members of the biofilm. Adherence can also be mediated via proteinaceous appendages, such as pili, fimbriae and flagella, protruding from the cell surface [102]. These events prime the early development of the biofilm architecture, Stage 3, during which other cells may be recruited into the biofilm either actively or passively. As the biofilm matures, Stage 4, channels and pores develop, and bacteria become redistributed, resulting in differences in gene expression across sub-regions of the biofilm [103, 104]. Biofilm maturation is a complex process that entails a number of social interactions and physiochemical variations that ultimately affect the structural architecture [105]. Eventually, the biofilm begins to disintegrate or detach, Stage 5, where cells disperse (either individually or in groups) either by fluid flow shearing off cells, or active processes on the part of the bacteria, reiterating the cycle in another location.

The main component of a biofilm is the matrix. Cells typically produce and embed themselves in a matrix of extracellular polymeric substance (EPS) composed of polysaccharides, proteins, nucleic acids, lipids and other biopolymers [95, 106, 107]. This matrix accounts for over 90% of the biomass and provides the biofilm with most of its properties [108]. The matrix provides a three-dimensional structure that allows the bacteria to carry out a wide range of functions such...
as adhesion, fortification, retention of water, horizontal gene transfer, enzymatic activity, utilization of new nutrient sources and absorption of inorganic ions. However, much of our knowledge of biofilm structure is extremely subjective, mainly because the biofilms generated in laboratories are highly dependent on the specific experimental setups utilized. Therefore, whether or not all biofilms grown in vitro are representative of what occurs in natural settings is up for contention.

**Figure 6.** Diagram showing the development of a biofilm under continuous flow conditions. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered “irreversible” attachment. Stage 3: early development biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. (Reproduced from Stoodley, P. et al. (2002). Biofilms as Complex Differentiated Communities. Annual Reviews in Microbiology 56, 187–209.)

**SEPTIC THROMBOSIS**

Medical devices have become indispensable in modern medicine. Catheter insertion is now widely accepted in clinical practice for the administration of fluids, blood transfusions, medications, nutritional support, chemotherapy and hemodynamic monitoring [109-112]. Most hospitalized patients have an intravenous catheter (IVC), and up to 30% have a central venous catheter (CVC) for long-term venous access with continuous perfusion or for patients with complicated peripheral venous access that require installation into a central vein [110, 113]. However, these devices are a major source of healthcare-associated infections, and the impact of these infections is substantial, both in terms of morbidity and financial resources expended [114-117]. The Center for Disease Control and Prevention estimates that 80,000 catheter-related bloodstream infections (CRBSIs) occur among patients in U.S. intensive care units (ICUs) alone [112], with approximately 250,000 cases occurring annually when data from the entire hospital are included [118, 119]. The outcome for CRBSIs is generally better when the etiological agents are coagulase-negative staphylococci. This is because they are more easily eradicated and are less pathogenic than coagulase-positive *Staphylococcus aureus* [117, 120-123]. *S. aureus* is the leading cause of bacteremia, with an annual incidence rate of 4.3 to 38.2 cases per 100,000 person-years.
in the United States [124]. The thirty-day all-cause mortality of *S. aureus* bacteremia is between 10 to 30% and has not changed since the 1990s [125]. The most prominent risk factor for invasive staphylococcal infection and bacteremia is prosthetic devices such as CVCs and surgically implanted materials that allow *S. aureus* to access the bloodstream. There are three recognized routes for the development of CRBSIs (Fig 7): 1) Extraluminal spread – migration of skin microflora (from the host or hospital staff) at the insertion site into the cutaneous catheter tract and along the surface of the catheter with colonization of the catheter tip; this is the most common route of infection for short-term catheters [122, 126, 127]; 2) Intraluminal spread – direct contamination of the catheter or entry port by contact with hands of hospital staff or contaminated fluids or devices [128, 129]; associated with both short- and long-term catheters; intrinsic contamination of infusates is rare [130]; and 3) Hematogenous spread – catheters become seeded from a distant focus of infection; this is less common [131].

![Figure 7. Diagram showing the possible routes of contamination that result in bloodstream infections.](image)

An intrinsic problem with IVCs is that they serve as a direct conduit into the intravascular space, where shortly after insertion, they become coated with a conditioning film of platelets, red blood cells, and extracellular matrix (ECM) components such as albumin, fibrinogen, fibrin, fibronectin, and laminin [132]. These host factors form a sheath around the catheter and prime the vasculature for microbial colonization and biofilm formation [133]. There is a close association between thrombosis of CVCs and infections. Septic thrombosis is an acute clinical complication that can result in the obstruction of blood flow due to a thrombus (blood clot) generated in the event of bacteremia, for which *S. aureus* is one of the primary causative pathogens [134]. However, the manifestation of a thrombus requires the conversion of host fibrinogen into fibrin. Therefore, anticoagulants (e.g. heparin) have been used to prevent thrombosis and presumably reduce the risk of infection. So why do clinicians observe worse patient outcomes associated with *S. aureus* colonization?

Heparin is a naturally occurring anticoagulant produced by basophils and mast cells [135], and is a member of the glycosaminoglycan family of carbohydrates. Therapeutic heparin is sold in either unfractionated or low-molecular-weight form and is the second most frequently used
natural drug that grosses $3 billion in sales annually. The anticoagulant effect of heparin is mediated by its interaction with the serpin antithrombin – the primary inhibitor of the blood coagulation proteases [136]. Heparin binds antithrombin, activating it through an allosteric modification and an increase in the flexibility of its reactive site loop [137, 138]. The heparin-accelerated inhibition of thrombin and factor Xa stops proteolytic cleavage of fibrinogen to form the fibrin clot. Patients with CVCs generally receive heparin prophylaxis (e.g. 3 units/mL in parenteral nutrition, 5,000 units every 6 to 12 hours flush or 2,500 units low-molecular-weight heparin subcutaneously) [139]. However, the drawback of this prophylactic measure in the event of an *S. aureus* infection is that the activity of the ProT•Coa or ProT•vWbp complex is not inhibited by heparin. *S. aureus* is still able to usurp the coagulation cascade, thereby causing exuberant, uncontrolled polymerization of fibrin without activation of other clotting and inflammatory factors. Therefore, ‘traditional’ anticoagulants do not prevent *S. aureus*-induced coagulation of blood and plasma. This is evident in our study [Manuscript 2 and 4], where *S. aureus* is still able to effectively generate clots and form a biofilm that is primarily composed of the host-derived matrix (HDM). It is this HDM that we believe to be a more accurate representation of the biofilms observed in vivo [Manuscripts 1 and 2].

Traditionally, the biofilm structure appears to be largely determined by the production of EPS, which provides structural support for the cells. However, bacteria do not exist in large patches or form the ‘mushroom-like’ structures as observed with some *in vitro* biofilm models. They rather exist as small aggregates that are interspersed throughout the host tissue and adhered to the ECM components that are deposited/recruited at the site of infection [Manuscripts 2, 3 and 4][140]. We see that *S. aureus* is able to form an intricate architecture of HDM by secreting coagulases [Manuscripts 2, 3, and 4]. Therefore, is a self-secreted EPS really the defining feature of biofilms *in vivo*? I propose no. The biofilms formed during infection can be thought of as an analogy: imagine an impending storm; one would rather build a shelter by utilizing the already available timber than to expend one’s own energy and resources in cutting down trees. In doing so, one bypasses the need for having to self-produce the timber and can then divert all other activity towards weathering the storm. In this analogy, the immune response and antimicrobials can be thought of as the impending environmental pressures that bacteria must face upon entering the host, and the ‘already available timber’ as the surrounding ECM components. This is the case with *S. aureus*, which secretes coagulases that allow it to utilize the already available host resources to build a fibrin shield around the bacterial community, protecting it from antimicrobials and infiltrating host immune cells [Manuscripts 2, 3, and 4].

One of the most prominent questions amongst clinicians and evolutionary biologists is to what extent are these social evolution theories relevant for infections? We address this by employing a streamlined bottom-up approach to explore the mechanistic and evolution theories surrounding *S. aureus* coagulases. We begin with a highly relevant *in vitro* clinical model, the findings from which are further tested in *ex vivo* human blood samples, and finally, the clinical significance of these theories are then verified in the more complex murine models of infection. Our *in vitro* biofilm model, termed ‘wound-like media’ (WLM), is actively used in clinical practice to reconstitute microbial populations from human infections such as chronic wounds. The applications, advantages, and financial incentives of using this model are the primary focus of
Manuscript 1. In addition, the review demonstrates why clinicians should use laboratory biofilm models that contain physiological components, such as serum or blood, when assessing in vitro biofilm data. In relation to that, the WLM is a relatively inexpensive model that enables us to mimic physiological conditions in the laboratory for rapid subjective evaluation of biofilm formation, population dynamics, and parallel assessment of tolerance to various antimicrobials in relation to our public goods of interest [Manuscripts 1, 2 and 4].

ANTIMICROBIAL TOLERANCE

In the matter of antibiotics and microorganisms, a bleak future awaits humanity on the near horizon; already infections that were treatable in the past have now become fatal. While we exhaust available antibiotics as our possible treatment options, we look towards understanding the social dynamics that transpire during infection in hopes of developing novel therapeutic strategies. The ESKAPE pathogens ( Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) are the leading cause of nosocomial infections throughout the world. Most of them are multidrug-resistant isolates and often associated with biofilms, which is one of the greatest challenges in clinical practice. Inoculation of calcium-chelated rabbit plasma is one of the primary diagnostic and identification techniques employed by clinical labs to differentiate S. aureus from coagulase-negative Staphylococci (CoNS) [16]. Despite this unique ability to generate robust fibrin clots, the antibiotic tolerance profiles of S. aureus clinical isolates are assessed using standard liquid cultures or agar plates [Manuscript 1]. S. aureus and P. aeruginosa can easily be 1000 times more tolerant to antibiotics when they are present in a biofilm in comparison to their planktonic state [141, 142]. This emergent property is what differentiates tolerance from resistance. Much of the tolerance profiles of biofilms can be attributed to the matrix itself that acts as a physical barrier, preventing certain antimicrobials from accessing the intended bacteria [Manuscripts 2 and 4]. It is likely that S. aureus binding to fibrinogen and the subsequent fibrin coat are defense strategies that also confer increased tolerance to antibiotics [Manuscripts 2 and 4]. The penetration of antimicrobials depends on the fundamental physical property of charge, where they experience electrostatic attraction or repulsion in the presence of the biofilm matrix. Most antibiotics are cations, anions, zwitterions, or a mixture; therefore, their diffusion rate depends on the environmental pH and the overall net charge of the biofilm matrix [143]. The heterogenic growth pattern within polymicrobial biofilms can also contribute towards the poor efficacy of antibiotics. The cells in the lower layers of the biofilm adopt a dormant or slow-growing lifestyle, therefore antibiotic that target metabolically active cells are ineffective against the inactive sub-population [144-146]. It is perhaps in this sense that antimicrobial tolerance has been the main driver in understanding biofilm-related infections. Therefore, not only do we assess the contribution of coagulases towards the antimicrobial tolerance profiles of S. aureus and P. aeruginosa, but also their benefit during infections [Manuscripts 2, 3 and 4].

COMPLEMENT SYSTEM & PHAGOCYTES

The innate immune system is the first line of host defense that plays a crucial role in the early recognition of pathogens, and in triggering a proinflammatory response during infection. The complement system is comprised of more than 30 types of soluble proteins that provide a highly
rapid and specific response against invading pathogens [147]. The proteins are abundant in blood serum and are synthesized continuously by hepatocytes, tissue macrophages, and blood monocytes. The system first evolved as part of the innate immune system and is complementary to the antibody response of the adaptive immune system, making complement a multifaceted and robust effector that bridges the innate and adaptive immune systems. The recognition of self and activation of the complement cascade occurs by any one, or more, of three biochemical pathways: the classical complement pathway, the alternative complement pathway, and the lectin pathway. The nomenclature of complement proteins is often an obstacle to understanding this system; therefore, before discussing the cascade, we will briefly clarify the usage of terms here: The components of the classical pathway and the membrane-attack complex (MAC) are designated the letter ‘C’ followed by a number, e.g. C1 and C3. The components of the alternative pathway are designated by capital letters instead of numbers, e.g. factor B and factor D. The products of the cleavage reactions of both pathways are identified by added lower-case letters, the larger fragments being designated ‘b’ and the smaller ‘a’; for example C4b and Bb. Please bear these in mind as we proceed.

The classical pathway is activated by the C1-complex, which is composed of three subunits designated C1q, C1r, and C1s. The pathway can be initiated by the binding of C1q directly to the pathogen surface, or by the binding of C1q to antigen-antibody complexes (Fig. 8). The lectin pathway is initiated by binding of the mannan-binding lectin (MBL) to mannose residues on the pathogen surface. The alternative pathway does not involve antibody activation but is rather initiated when a spontaneously activated complement component binds to the pathogen surface. The classical and lectin pathways split and then join fragments of C4 and C2 (C4b2b); whereas the alternate pathway utilizes fragments of C3 and factor B (C3bBb) to generate homologous variants of the protease C3-convertase (Fig. 8). These reactions are part of the ‘early’ events of complement activation. Here the C3-convertases cleave C3 to generate C3a, a peptide mediator of inflammation, and C3b that acts as an opsonin – it binds to pathogens and targets them for destruction by phagocytes. C3b can also form a C5-convertase that cleaves C5 into C5a, an important chemotactic protein, and C5b, which initiates the ‘late’ events of complement activation. These comprise a sequence of polymerization reactions in which C5b, C6, C7, C8 and C9 form the MAC. The cytolytic endproduct, MAC, creates a pore in the lipid bilayer membrane, which compromises the membrane integrity (Fig. 8). This is thought to kill the invading pathogen by destroying the proton gradient across its cell membrane.

<table>
<thead>
<tr>
<th>Activation Pathway</th>
<th>Classical</th>
<th>Alternative</th>
<th>Lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activator</td>
<td>Antigen-Antibody complex</td>
<td>Spontaneous hydrolysis of C3</td>
<td>MBL-Mannose complex</td>
</tr>
<tr>
<td>C3-convertase</td>
<td>C4b2b</td>
<td>C3bBb</td>
<td>C4b2b</td>
</tr>
<tr>
<td>C5-convertase</td>
<td>C4b2b3b</td>
<td>C3bBbC3b</td>
<td>C4b2b3b</td>
</tr>
<tr>
<td>MAC development</td>
<td>C5b+C6+C7+C8+C9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Table lists the similarities and differences of the classical, alternative, and lectin pathways of the complement system and the various constituents involved in each pathway.
Figure 8. The complement system and its regulatory steps involved in pathogen killing. (Reproduced from OpenStax, Innate Immune Response. OpenStax CNX. Dec 19, 2016 http://cnx.org/content/5da10a86-3129-460c-b5b6-0a40c00b9969@6.)

Opsonization by components of the complement system and/or immunoglobulins (Igs) is an important step for microbial clearance [56]. Complement activation is largely confined to the surface on which it is initiated; this ensures specificity. The binding of a large number of C3b molecules to the pathogen surface is the central event in complement activation. Pathogens
opsonized by C3b and its inactive fragments are recognized by specific complement receptors on phagocytic cells that ingest the complement-tagged pathogens. However, Staphylococcus aureus has means to generate a protective coat of polysaccharide, or fibrin that impedes phagocytic uptake of staphylococci by neutrophils or macrophages [148, 149]. S. aureus interfaces with complement activation by encasing itself in layers of fibrinogen or fibrin that prevent complement factors from effectively binding its surface and prevent MACs from penetrating the cell membrane. This is reflected in our study [Manuscripts 2 and 4] where access to coagulases significantly improved staphylococcal survival in human blood, an environment where complement killing plays a major role. Interestingly, this protection was conferred onto the cheats and Pseudomonas aeruginosa, and not just the producers of the public goods. Coagulases are not the only factors that enable S. aureus to diminish or delay opsonization. Many of its virulence factors can directly interrupt complement pathways. Staphylococcal complement inhibitor (SCIN) or the SCIN-B and SCIN-C homologs, and extracellular fibrinogen binding protein (Efb) or its homolog Ehp can block C3-convertases [150]. Its extracellular adherence protein (Eap) can bind to C4b, thereby preventing the formation of the fully active C3-convertase [151]. S. aureus secretes aureolysin, a metalloprotease that can cleave and inactivate C3 [152]; and recruits complement inhibitory factors H and I via clumping factor A (ClfA) and surface-anchored protein SdrE, which also cleave C3 [153]. Staphylococcal protein A (SpA) and staphylococcal superantigen-like 10 (SSL10) bind Igs and block the effector domain of the opsonizing antibodies [154-156]. Furthermore, the active staphylokinase (Sak)-plasmin complex cleaves the Igs and C3 that do manage to bind to the surface of S. aureus [157]. The host chemotactic protein, C5a, can also initiate a local inflammatory response and help recruit leukocytes; however, S. aureus targets complement factor C5 via the secretion of staphylococcal superantigen-like 7 (SSL7) [158].

In addition to these evasion mechanisms, binding to fibrinogen is an important attribute and determinant for S. aureus virulence during infection. Studies reveal that the essential requirement for antibodies to be efficient is their ability to inhibit the binding of fibrinogen to staphylococcal factors [159, 160]. S. aureus can decorate its surface with fibrinogen through other factors such as clumping factors (ClfA and ClfB), fibronectin binding proteins (FnbpA and FnbpB), and extracellular matrix binding protein (Empbp). It should be noted that, with the exception of coagulases, the ability of S. aureus to express all other virulence genes was a common factor in our study [Manuscripts 2, 3, and 4]. However, the interaction of these factors with the host extracellular matrix (ECM) does not precipitate fibrinogen cleavage and/or fibrin clot formation, that ability is reserved to Coa and vWbp [Manuscripts 2, 3, and 4]. As a result, the binding of ClfA, ClfB, FnbpA, FnbpB, and Empbp to fibrinogen alone is not enough to rescue the cheats or P. aeruginosa. Hence, the deposition of a large amount of fibrinogen is most likely a prerequisite for the subsequent development of fibrin coats. The C-terminal end, designated fibrinogen-binding domain, of Coa allows it to interact with at least four fibrinogen/fibrin molecules per Coa molecule to form a mega-protein complex around S. aureus [62]. In addition, vWbp further fortifies the bacterial community by strengthening the nascent fibrin clot with covalent crosslinks [63]. Therefore, our observations of staphylococcal defense against complement killing are with respect to our public goods of interest, coagulases, and the product of their enzymatic activity, fibrin [Manuscripts 2 and 4]. So far we’ve discussed
opsonophagocytosis and the cytolytic properties of MACs; however, not all pathogen recognition is opsonin dependent.

Phagocytes can also recognize the evolutionarily conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs) via germline-encoded pattern recognition receptors (PRRs) [161, 162]. PAMPs are characterized by being invariant among entire classes of pathogens and are essential for their survival [163]. Common PAMPs include peptidoglycan, flagellin, lipopolysaccharide (LPS), lipopeptides, and nucleic acids. As a general rule, the interactions between PAMPs and PRRs ensure specificity and the ability to distinguish ‘self’ (host cellular components) from ‘nonself’ (foreign material). There are several families of PRRs such as toll-like receptors (TLRs), complement receptor 3 (CR3), glycosylphosphatidyl inositol-linked membrane protein (CD14), scavenger receptors, mannose and glycan receptors. Many of these PRRs are expressed at the cell surface of the phagocyte, whereas others are located in intracellular compartments and organelles, including endosomes and lysosomes. Upon recognition of a PAMP, the PRR sends a signal to the nucleus of the phagocyte to elicit a response such as activation of genes involved in phagocytosis, cellular proliferation, production and secretion of antiviral interferon and proinflammatory cytokines, and enhanced intracellular killing [164]. The recognition of PAMPs is a critical event for the activation of the innate immune response. However, these bacterial structures also contribute towards constructing and stabilizing the overall biofilm matrix [94]. For example, 1) Proteinaceous appendages, such as pili, fimbriae, and flagella, are used to form cross-linking structures and filaments in biofilms [165, 166]; 2) Lectins provide a link between the pathogen surface and the EPS [108, 167]; and 3) Nucleic acids, such as extracellular DNA, are an important structural component of the biofilm matrix. Therefore, in the event of the HDM being formed by coagulases, many of these bacterial structures can become sequestered and/or integrated within the fibrin network, thus delaying pathogen recognition by interfering with the binding of PAMPs to PRRs. The importance of PAMP-PRR interaction for recruiting phagocytes was highlighted in a study where *S. aureus* variants lacking lipoproteins were able to escape host immune recognition and disseminate into host tissue with increased lethality during infection [168].

Neutrophils comprise 60–70% of human white blood cells, making them the most abundant polymorphonuclear leukocytes present in blood and a principal defense against *S. aureus*. In turn, the pathogen interferes with neutrophil recruitment via secretion of staphylococcal superantigen-like 5 (SSL5) and SSL10; chemotaxis inhibitory protein of *S. aureus* (CHIPS); and its homologs, formyl peptide receptor-like inhibitory proteins (FLIPr and FLIPr-like) [169-171]. Furthermore, *S. aureus* uses Eap to block the adherence of neutrophil ligands to cognate endothelial adhesion receptors [48], and SSL5 to prevent neutrophils from rolling on the endothelial cells [172], thereby interfering with the extravasation of leukocytes towards the infectious foci. However, in the event of arrival, neutrophils release neutrophil extracellular traps (NETs) comprised of neutrophil DNA, histones, proteases and antimicrobial peptides [173]. In response, *S. aureus* initiates its defense protocols by deploying Sak and aureolysin that inactivate antimicrobial peptides through direct binding or proteolytic cleavage; and further fortifies its cell wall envelope against muralytic enzymes and antimicrobial peptides through chemical modifications of peptidoglycan and synthesis of secondary cell wall polymers, including wall teichoic acid (WTA) and lipoteichoic acid (LTA) [174, 175]. In addition, Eap is a potent inhibitor
of neutrophil serine proteases such as elastase, proteinase 3, and cathepsin G. S. aureus also mounts a counterattack by secreting nuclease to degrade NETs to escape DNA entrapment and peptide defensins, and generate deoxyadenosine to trigger caspase-3-mediated death of immune cells [176, 177]. Despite the evasion attempts, phagocytes are still able to engulf the staphylococci and commence digestion via acidic vacuoles, reactive nitrogen and oxygen species. However, S. aureus copes with oxidative stress by producing staphyloxanthin, a membrane-bound carotenoid pigment that scavenges reactive oxygen species [178-181], whereas its other detoxifying enzymes can convert hydrogen peroxide to oxygen and water [182]. Furthermore, it can withstand the nitrosative stress by secreting flavohemoglobin, a potent neutralizer of reactive nitrogen species [183]. In doing so, S. aureus is able to survive within leukocytes for several days and employ a ‘Trojan horse’ approach, where it uses the professional phagocytes to extravasate and disseminate into uninfected host tissues (Fig. 9) [184].

ABSCESS ES

During the Ninth Surgical Congress in Berlin (1880), Sir Alexander Ogston presented his findings on the ability of staphylococci to generate abscesses in infected animals. Since then, Ogston’s microbe – Staphylococcus aureus, is now the most frequently associated etiological agent of suppurative abscesses. However, the formation of abscesses is not unique to S. aureus invasion. In principle, the events of abscess formation are part of a default host response that can be induced by the injection of biological material (infectious or sterile) into tissues, chemical trauma, or physical insult [185, 186]. Any one of these instances triggers a proinflammatory response mediated by the release of cytokines and chemokines from macrophages, neutrophils, and other immune cells at the site of injury and/or infection. The degradation of damaged tissues by polymorphonuclear leukocytes is accompanied by liquefaction necrosis and the deposition of fibrin to delineate areas of inflammation from healthy tissues; the localized pus is then drained to organ surfaces to promote healing [187-189]. Interestingly, this classical host-mediated response is also subject to being hijacked by S. aureus.

To dissect the relative contribution of coagulases from a social evolution perspective, we utilize an in vivo murine abscess model. The retro-orbital plexus (intravascular) route of delivery allows us to simulate the hematogenous spread of S. aureus, where the inoculum drains into the external jugular vein; which converges in the thorax with the internal jugular and the subclavian veins into the ipsilateral superior vena cava, achieving high blood levels rapidly [Manuscript 3][190]. As discussed previously, S. aureus blocks the innate defenses of its host upon entering the bloodstream. The various evasion mechanisms facilitate staphylococcal survival within the vasculature, which is followed by its entry into organ tissues, where it can seed abscess lesions. In addition to relying on professional phagocytes for extravasation and dissemination, S. aureus can utilize its arsenal of adhesins/invasins to exit the bloodstream, the most prominent of which may be fibronectin binding proteins (FnbpA and FnbpB). The fibronectin-binding repeats of Fnbps engage multiple fibronectin molecules, which are recognized by the α5β1 integrin of endothelial cells [191, 192], triggering bacterial uptake/transmigration via a host-cell driven process [193, 194]; this is believed to facilitate bacterial persistence and the establishment of secondary (metastatic) infections [195-197]. The wall teichoic acid (WTA) and lipoteichoic acid (LTA) of S. aureus have also been implicated in promoting staphylococcal invasion of host cells [174, 198,
Another important route of escape may be the disruption of the physiological endothelial barriers. *S. aureus* α-hemolysin (Hla) binds to the host receptor ADAM10 (A-disintegrin and metalloprotease), upregulating the cleavage of vascular endothelial cadherin, thereby compromising the endothelial barrier and promoting vasculature leakage for entry into host tissues.

The initial introduction of *S. aureus* into the vasculature is followed by a precipitous drop in the staphylococcal load from the bloodstream and accompanied by an increased bacterial burden in multiple organ tissues (Stage 1) [200]. Staphylococcal invasion of host tissues triggers a massive infiltration of immune cells, responding to cellular destruction and proinflammatory signals at the site of infection. Despite their recruitment, even under these circumstances, only very few of the leukocytes are able to phagocytize the staphylococci. As the infection progresses to Stage 2, a low abundance of staphylococci and a large number of immune cells (mainly neutrophils) are observed at the infectious foci, with no discernable organization. The next 48-72h promotes abscess maturation; the staphylococcal abscess communities (SACs) located at the center of the lesion become enclosed by fibrin deposits and are surrounded by concentric layers of immune cells [200]. During Stage 3, the lesions convert to a complex structure, where the SACs are surrounded by a cuff of immune cell infiltrates comprised of three layers of neutrophils that are apoptotic, healthy, and apoptotic in appearance, respectively; followed by a layer of other immune cells such as macrophages and lymphocytes at the lesion periphery [177, 200]. The distinct eosinophilic (readily bind the eosin dye) fibrin deposits demarcate abscess lesions from the healthy, uninfected tissues, but can also inadvertently serve as a shield against infiltrating immune cells [Manuscript 3][200]. By day 15 of post-infection, the abscesses are fully mature, where the SACs have expanded in size, all the while remaining enclosed in an eosinophilic capsule; the renal tissue architecture is now replaced by necrotic immune cells and accumulating cellular detritus (Fig. 9) [Manuscript 3][200]. Eventually, the abscesses rupture and release into the peritoneal cavity, initiating a new infectious cycle (Stage 4).

It should be noted that the stages of abscess formation have been established in previous studies [200, 201]; one of which examined the virulence consequences associated with sortase A, a transpeptidase that anchors a large spectrum of proteins to the cell wall envelope of *S. aureus* [200]. The study demonstrated that heme scavenging factors (IsdA and IsdB); as well as serine-aspartate-repeat protein D (SdrD) and protein A are necessary for abscess formation [200]. IsdA and IsdB enable *S. aureus* to meet its nutritional requirements (iron acquisition) for expansive growth during these stages; whereas, SdrA contributes towards counteracting neutrophil defenses [202]; and protein A impedes phagocytosis by binding the functional component of immunoglobulins (Igs) [203, 204]. They also found that clumping factors (ClfA and ClfB) are required during the early phase of staphylococcal dissemination, but are not required for abscess formation. However, here we illustrate the stages of abscess development to contextualize how cheats are integrated into the abscess community created at the expense of the producers (Fig. 9) [Manuscript 3]. This model highlights that our public goods of interest, coagulases, facilitate the metamorphosis of the SACs by usurping the clotting cascade and forming fibrin deposits in organ tissues (Fig. 9) [Manuscript 3]. These fibrin deposits surround the SACs and function as a barrier against invading immune cells and prevent their penetration into the SACs. In addition to helping the producers escape phagocytic clearance, the two coagulases also contribute to the
survival of the cheats that reside within the abscess community by concerting the formation of distinct layers of fibrin described above (Fig. 9) [Manuscript 3]. This allows both producers and cheats to replicate without interference while manipulating the host inflammatory responses, the fate of the immune cells in the periphery and the progression of the entire infectious lesion from a safe distance.

Figure 9. Working model for how cheats survive within staphylococcal abscesses generated by producers. Stage 1: following intravenous infection, S. aureus wt and mutants survive in the bloodstream and disseminate into host tissues via passive blood flow or taxing in host immune cells. Stage 2: polymorphonuclear leukocytes and other immune cells (in blue) begin to arrive at the site of infection. Stage 3: abscesses mature with a central staphylococcal abscess community (SAC) now enclosed by a wall of fibrin shields. The SAC becomes surrounded by dead host immune cells (in grey). (Adapted from Cheng et al. (2009). Genetic requirements for Staphylococcus aureus abscess formation and persistence in host tissues. The FASEB Journal 23, 3393–3404.)
Undoubtedly the microscopic images of the septic thrombi resemble biofilm growth, where one can see that the microcolonies are embedded within a network extracellular matrix components [Manuscripts 2 and 4][140]. The WLM model is set to mimic the physiological components encountered during infections [Manuscripts 1, 2, and 4]. In relation to that, many of the constituents involved in thrombosis, such as fibrinogen and fibrin, are also involved in abscess development. However, despite the commonality, we propose that the ability of S. aureus to elicit the complex architecture of abscess lesions is best appreciated as a series of complex interactions between the pathogen’s virulence factors and the molecules, cells, and tissues of its host; it should not be mistaken for biofilm growth.

**CHRONIC WOUNDS**

The role of biofilms is better appreciated in a multitude of chronic infections such as those involving wounds, catheters, stents, surgical implants, endodontic infections, and cystic fibrosis [205-211]. The National Institute of Health (NIH) estimates that biofilm phenotype bacteria cause 80% of all human infectious diseases and that list continues to expand [212, 213]. However, an in-depth discussion on all biofilm-related infections may prove to be beyond the scope of this thesis. Instead, we will focus on one – chronic wounds, which have become a rising problem in conjunction with comorbidities such as obesity and diabetes. Biofilms are involved in more than 90% of all chronic wound infections [214], which more often than not harbor more than one species of bacteria, of which *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the two organisms most frequently found together and isolated from the wounds of patients. Chronic wounds can stem from venous leg ulcers, diabetic foot ulcers, pressure ulcers, physical insult and trauma injuries that often go unresolved due to their recalcitrant nature and failure to heal. There are multiple factors that contribute towards chronicity, of which antibiotic tolerance alone cannot explain why chronic wounds are not cleared by the host immune defense.

The immune system uses a complex system of proinflammatory cytokines including tumor factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and interferon-alpha (IFN-α). The upregulation of proinflammatory cytokines is an almost universal physiological characteristic of chronic wounds. When functioning properly, these cell-signaling proteins can coordinate recruitment, activation, and subsequent removal of professional phagocytes [215-218]. In contrast to these physiological regulatory steps, pathogens entrenched in biofilms can commandeer the host immune response as part of their survival mechanism. *S. aureus* and *P. aeruginosa* can overwhelm the PRRs of immune cells by generating an overabundance of specific PAMPs and effectively overrun the expression of the otherwise tightly controlled pro-inflammatory cytokines.

The necrotic disintegration of neutrophils *in situ* is prevented by macrophages that recognize engulf, and degrade the functionally terminal neutrophils. This orderly elimination of neutrophils by macrophages is mediated by cellular signaling between the two immune cells. However, the overabundance of proinflammatory cytokines and PAMPs can interrupt the communication between the phosphatidylserine (PS)-bearing membranes of neutrophils and macrophages, causing the neutrophils to become trapped. The over-accumulation of neutrophils and their untimely apoptosis results in a surge of proteolytic enzymes such as elastase, collagenase,
gelatinase, metalloprotease, and inflammatory mediators at the infectious locale (Fig. 10) [219-221]. In turn, these enzymes damage the surrounding host tissue, red blood cells and immune cells that should otherwise be involved in the wound repair process [222]; this leads to yet an even further production of additional pro-inflammatory cytokines, stimulating a persistent influx of inflammatory cells and plasma that provide the biofilm with a continuing nutrient source. Therefore, by actively recruiting and killing immune cells, S. aureus and P. aeruginosa can induce a state of perpetual inflammation that overwhelms the anti-protease defenses and maximizes the damage to host tissue, and thereby promoting chronicity (Fig. 10).

Figure 10. Biofilm maintenance: once the bacteria have attached, established a protected community, organized themselves within the tissue and subverted host cellular function, they maintain a sustainable inflammatory niche by manipulating the host immune response. Chronic wounds contain excessive neutrophils. Interleukin-8 receptor (CXCR-1), which is bound within the neutrophil’s membranes, activates the neutrophil and causes it to migrate into the wound (J). Many bacteria possess virulence factors that kill neutrophils (K). If they are not engulfed by macrophages, the dying neutrophils release large amounts of elastase into the environment. This neutrophil-derived elastase degrades the CXCR-1 receptor on neutrophils, causing them to stagnate and preventing them from being properly phagocytized by macrophages. The fragments of the CXCR-1 receptor stimulate Toll-like receptor-2 (TLR2) on dendritic cells and macrophages to produce a massive release of proinflammatory cytokines (L). The action of pathogen-associated molecular patterns (PAMPs) and fragments of CXCR-1 work through a nuclear factor Kappa B pathway (NF-κB) to release proinflammatory cytokines (M). Tissue inhibitors of matrix metalloproteases (TIMPs) produced by the wound bed cells are deficient and do not downregulate the production of proinflammatory cytokines. The proinflammatory cytokines create gaps in the endothelial cells of the capillaries, allowing neutrophils to migrate through the vessel wall into the wound bed (diapedesis). This completes a sustainable cycle in which bacteria invoke excessive neutrophil migration and cause persistent inflammation (Reproduced from Wolcott, R.D. et al. (2008). Biofilms and chronic wound inflammation. Journal of Wound Care 17, 333–341.)

Immune cells are an integral part of both abscess and chronic wound development. However, a notable feature of S. aureus and P. aeruginosa infections is that the dramatic infiltration of leukocytes is not strictly host-mediated. In contrast to interfering with neutrophil recruitment, S. aureus is also able to orchestrate the recruitment of immune cells by releasing proinflammatory molecules such as lipoproteins that are recognized by the innate immune toll-like receptor-2 (TLR2) [168]. In addition to this, S. aureus can stimulate neutrophil chemotaxis to the site of infection via secretion of formylated phenol-soluble modulins (PSMs), as part of its pathogen-
driven developmental process, aiding staphylococcal replication and dissemination. *P. aeruginosa* also utilizes such elaborate strategies during infection where it can limit the host inflammatory response by downregulating its synthesis of flagellin that is recognized by the innate immune TLR5 [223, 224]. In addition, *P. aeruginosa* has been shown to produce a number of molecules that directly inhibit or are toxic to immune cells. Rhamnolipid, a glycolipid surfactant, acts as a shield against infiltrating leukocytes and causes neutrophil necrosis [225, 226]; pyocyanin, a redox-active phenazine, can trigger neutrophil apoptosis [227]. In contrast, *P. aeruginosa* can actively recruit immune cells by expressing a highly acylated lipid A variant of lipopolysaccharide that is a strong TLR4 agonist. The infiltrating immune cells then commence fighting the infection through oxidative burst and nitric oxide production. In doing so, the neutrophils consume a sizable amount of oxygen for their production of reactive oxygen species and nitric oxide [228, 229]; however, since they cannot penetrate into the staphylococcal abscess communities (SACs) and biofilms, the surrounding host cells are robbed of the bulk oxygen available for respiration. These mechanisms allow *S. aureus* and *P. aeruginosa* to strike a balance between blocking their own clearance from phagocytes and recruiting cells to develop infectious lesions for their dissemination to new sites.

We find the contribution of coagulases towards bacterial persistence in chronic wounds to be minimal [Manuscript 4]. The fitness of *P. aeruginosa* and cheats was independent and relatively unaffected by whether or not they had access to coagulases. This can perhaps be explained by the fact that *P. aeruginosa* itself is already highly specialized towards establishing biofilms in chronic wounds. Therefore, whatever the initial advantages may have been of having access to coagulases are overshadowed by the already well-established biofilm in the wounds at later time points. The extracellular adherence protein (Eap) of *S. aureus*, on the other hand, maybe a more relevant candidate for studying social interactions occurring in chronic wound infections. These infections involve a high degree of inflammation where the bacteria have to cope with recurrent attacks from immune cells. In that sense, it appears Eap may interfere with the immune response by binding to and inhibiting neutrophil serine proteases [230], as well as reducing neutrophil recruitment to the site of infection and altering the development of adaptive immunity [48, 231, 232]. Re-epithelialization and tissue granulation in context with neovascularization are important steps of the wound-healing process. However, Eap can also block the αv-integrin–mediated endothelial-cell migration and capillary tube formation, and neovascularization in matrigels in vivo [233]. Collectively, the anti-inflammatory and antiangiogenic properties of Eap explains how this protein may be engaged in the pathogenesis of impaired wound healing in wounds chronically infected with *S. aureus* or ulcers. Collectively, Eap may be able to function as a public good during the establishment of chronic wound infections, making it an excellent candidate for future studies. Despite not having observed any advantages within the wounds, we do observe the bacterial load in splenic tissue corresponds to whether or not the bacteria had access to coagulases [Manuscript 3 and 4].

**SECONDARY INFECTIONS & SPLENIC TISSUE**

The ability of the innate immune system to recognize and limit microbes during the early stages of infection relies primarily on complement activation, phagocytosis, autophagy, and immune activation. These early events culminate in the activation of the adaptive immune response, that
can act in concert with the innate immune system to increase the killing efficiency through the recruitment of cell-mediated and antibody responses. There is a degree of overlap between the two systems that covers the temporal lag between the immediate response and the development of antibodies. This intermediate response is provided by the B1 cells in the body cavities and the spleen, which produce antibodies of a limited repertoire [234]. The white pulp of the spleen contains T and B lymphocytes, natural killer cells, and antibodies such as IgM and IgG2 that play a role in opsonization of extracellular organisms. Whereas the red pulp of the spleen houses over half of the body's monocytes that later differentiate into macrophages and dendritic cells while promoting healing [235, 236]. The spleen removes antibody-complexed pathogens and antibody-coated blood cells by way of blood and lymph node circulation. It also allows antigen presenting cells (APCs) that have trapped foreign particles in the blood to communicate with lymphocytes. Therefore, how do we justify our observations regarding splenic tissue in our murine abscess and chronic wound models?

As the abscesses continue to mature, T and B lymphocytes, as well as macrophages, infiltrate lesions, which are slowly pushed towards the organ surface (e.g. renal cortex and renal capsule), where they eventually rupture and release purulent exudate and staphylococci for reentry into the bloodstream or dissemination to new sites of infection. The fibrin fibers derived from the ProT•Coa and ProT•vWbp complexes may promote endothelial adherence by inducing microthrombi that have bacteria embedded within a fibrin meshwork [237]. In turn, the septic thromboemboli obstruct blood flow in the microvasculature and provide the bacteria with a possible route of invasion into organ tissues. This mechanism of bacterial spread may be correlated with the previously discussed clinical phenomenon of disseminated intravascular coagulation associated with *S. aureus* bloodstream infection and sepsis [238]. This mode is not confined to abscesses; we propose a similar mechanism of systemic spread occurs with chronic wound infections where coaggregates of *S. aureus* and *P. aeruginosa* seed back into the bloodstream and disseminate to new host tissues as septic thromboemboli. The Coa and vWbp mediated microthrombi provide an explanation for the increased bacterial load in the splenic tissue, whereas another explanation is the ability of *S. aureus* and *P. aeruginosa* to survive inside the immune cells. Since the spleen functions to immunologically filter the blood and allow for communication between cells corresponding to the innate and adaptive immune responses. The bacteria that manage to survive within phagocytes after being engulfed are carried into the spleen; in this sense, the host immune cells, unknowingly, act as a vehicle for infection spread. In addition to that, bacterial persistence and a perpetual state of inflammation at the site of infection results in the spleen having to produce an abnormal amount of red blood cells, antibodies, monocytes, and lymphocytes in an attempt to clear the infection. Subsequent to which, these large numbers of immune cells need to be filtered out of the blood. This leads to an accumulation of bacterial-harboring immune cells within the spleen. Taken together, all of these factors most likely contribute towards the clinical phenomenon of splenomegaly (enlarged spleen) observed in our model [Manuscript 3]. However, ultimately, we find that coagulases are the public goods that facilitate increased bacterial persistence within the host bloodstream and organ tissues [Manuscripts 2, 3, and 4].
The ability of *Staphylococcus aureus* to clot blood and plasma was first reported in 1903 and has, since then, become one of the primary diagnostic markers for differentiating this pathogen. The significance of *S. aureus*-induced coagulation is evidenced by the fact that clinical laboratories around the globe still utilize rabbit plasma in which isolates from patients are inoculated to positively identify *S. aureus*. This suggests a worldwide distribution of Coa and vWbp among *S. aureus* strains. Coincidentally, the widespread use of coagulation tests has also led to the discovery of atypical coagulase-negative strains that are found together with coagulase-positive *S. aureus* strains. Our understanding on their co-occurrence in the clinics is somewhat lacking, but here we provide an explanation supporting the benefits of residing with a Coa- and vWbp-producing strain from a social evolution perspective and why a deviation from this trait occurs during infection. This is not only of interest to clinicians, but also sheds light on the social interaction aspects of this fascinating trait of *S. aureus*, and why it matters during polymicrobial infections.

The search for such novel public goods, however, requires that we shift away from the various standard laboratory growth media and employ models representative of physiological conditions (blood, serum, host immune cells and epithelial cells). These growth conditions may not be the preferred source of nutrients, but they do reflect what pathogens encounter during infections. Without such models, it will be difficult to identify and screen other potential public goods that contribute towards bacterial pathogenesis. Consider standard laboratory growth media where there is an excess of resources such that a trait of interest provides no benefit nor plays a role, leading one to conclude that the trait is not beneficial or exploitable, which would be an error, when it in fact is, just not in that particular environment. To give an analogy, this would be the equivalent of testing how a wolf pack hunts collectively as a group, but carrying out the study in a zoo, where there was *ad lib* food and there is no need to hunt. This is well depicted by the fact that clotting, a trait associated with coagulases or metalloproteases, can be observed in the presence of plasma and fibrinogen, but not in conditions without. Therefore, appropriate environments are needed in obtaining a detailed molecular understanding of such traits and elucidate their specific role and contribution during infections.

A first study on the social nature of coagulases in their natural habitat has provided unique insights from the perspective of both clinical sciences and evolution biology. These social theories should also be applied towards studying the clotting ability of *Yersinia pestis*, which produces its own version of coagulase. In addition, *Escherichia coli*, *Bacillus cereus*, and *Bacillus anthracis* are also known to activate human and mouse coagulation factors. Which begs the question: Given its costly nature and benefits for microbes, why is this trait not a subject of enforcement (repression of competition or policing)? Perhaps the lack of enforcement regarding coagulation can be explained by the observation made in *B. anthracis*, which secretes a zinc metalloprotease InhA1 to activate the host coagulation factors. However, for *B. anthracis*, this trait does not require a change in gene expression; it can be rapid and independent of bacterium-to-bacterium communication, making coagulation a ‘quorum acting’ trait rather than a quorum sensing trait. However, whether or not this applies to all other coagulation-inducing microbes needs to be investigated.
The observations and the methods employed in this thesis can be applied towards discovering novel drug candidates that directly target public goods for improving the already available treatment rubric. For example, we demonstrate that the matrix generated by coagulases hamper the efficacies of antimicrobials. Therefore, along with targeting the bacteria, we suggest that clinicians should also target the mechanisms of *S. aureus*-mediated coagulation. Although the traditional anticoagulants used in the clinics do not prevent *S. aureus*-induced coagulation, the use of direct thrombin inhibitors, such as dabigatran and argatroban have shown appreciable potential against *S. aureus* in animal models. In that sense, perhaps inhibitory doses of such direct thrombin inhibitors in concert with antimicrobials could result in better prognosis and overall clinical outcomes. However, not all potential goods of *S. aureus* should be the targets of treatment, when in fact some can be isolated in their enzyme form and used as treatments instead.

Of the various virulence factors that *S. aureus* secrete, lays a fascinating anti-inflammatory protein, extracellular adherence protein (Eap), whose role as a potential public good needs to be determined in a wide variety of infections. *S. aureus* uses Eap as part of its immune evasion strategies to inhibit leukocyte recruitment. If you recall, bacterial invasion, growth, and persistence in the body are multifactorial, where inflammatory response plays a major role but is not the deciding factor for all types of infections. However, as described in the chronic wounds section of this thesis, unregulated adhesion of leukocytes and/or endothelial cells results in uncontrolled cellular extravasation and recruitment of neutrophils that causes a persistent state of inflammation. In such pathological processes, where patients present hyperinflammatory pathologies, Eap could be used as a therapeutic substance to design new peptides or non-peptidic molecules that block leukocyte extravasation and induce anti-inflammatory responses. The future studies envisioned are just a few examples that highlight the critical need and potential for dissecting the molecular mechanisms of such bacterial produced public goods and exploiting their role in treating a wide variety of infections.
This chapter illustrates some of the basic concepts of phenotypic plasticity, genetic assimilation, and bet-hedging. A discussion of these topics extends into the main focus of this part of the introduction: how and why phenotypic plasticity is advantageous during the lifespan of an individual? We address this question using our model organism – *Pseudomonas aeruginosa*. This gram-negative opportunistic pathogen is known for its diversity in morphotypes and niche occupation. The almost universally associated phenotypes with *P. aeruginosa* are: 1) Biofilm formation; 2) Flagella-mediated motility; and 3) The ability to cause a wide variety of infections. Therefore, given its widespread distribution and capabilities suggests a high degree of physiological and genetic flexibility within this ubiquitous pathogen. Here we will provide a short overview of c-di-GMP, which is a secondary messenger and has been the research subject of Manuscript 5 in this thesis. Hence, this short section will focus on the general influence of c-di-GMP in directing bacterial behaviors such as motility, biofilm formation, and its potential of coordinating responsive switching in *P. aeruginosa*. Lastly, we provide a short summary of our findings and engage the readers in a short discussion of the supplementary results generated for future studies.
All of life is flux; the only thing constant is change. The aim of life-history theory is to explain the diverse characteristics of organisms – including anatomy and behavior – that have been shaped by natural selection, genetic drift, or other mechanisms of organic evolution. The extent of diversity between different species is obvious, but how does one explain the great diversity in life-history characteristics within two individuals of the same species? Undoubtedly, the genetic background of individuals plays a role in their life-history traits, where genetic differences account for differences. However, an organism’s characteristics are not solely dependent on its genetic inheritance; its environmental, demographic and social conditions can also influence its life-history traits. For example, a mustard plant seedling exposed to high levels of carbon dioxide will develop leaves with fewer stomata in comparison to when it is grown in a low carbon dioxide environment [239-241]. This difference is an example of ‘phenotypic plasticity’ (responsive switching) – the property of a given genotype to produce different phenotypes in response to distinct environmental conditions [242].

The concept of phenotypic plasticity has been at the center of the age-old question of nature versus nurture, occupying philosophers from Plato to Locke [242]. Its relationship with the genetics of organisms has been a topic of great discussion within the context of modern evolution theory. A general consensus is that plasticity can be selected if organisms are faced with a relatively high degree of environmental fluctuations. However, it should be clarified that phenotypic plasticity does not necessarily improve the organism’s survival and reproduction. Thus, in forgoing the parsimonious sense of adaptation, some traits simply evolve due to the constraints imposed by the biochemistry, physiological or developmental biology of the organism [243]. The role of plasticity in evolution should not be mistaken to be on par with natural selection, plasticity is a proximate cause of changes in a trait, while natural selection is an ultimate cause of adaptation during evolution, where selection acts on plasticity. This can lead to genetic assimilation – a process by which what was once an environmentally induced phenotype becomes genetically encoded and no longer requires the original environmental signal for expression.

Since its introduction during the late 19th century, the topic of genetic assimilation has also been of great interest within the context of modern evolutionary theory [244-248]. However, there hasn’t always been an agreement regarding the ecological importance of plasticity and genetic assimilation [249, 250]. After clearing the fog, one finds that much of the controversy stems from conceptual confusion and a misunderstanding of how to two actually fit into modern evolution theory. The significance of one concept does not deny the importance of the other, where in fact, genetic assimilation is actually linked to phenotypic plasticity:

“Plasticity is what makes possible the appearance of an environmentally induced novel phenotype, and a process of selection on the expression of such phenotype in a new environment may end up ‘fixing’ (genetically assimilating) it by altering the shape of the reaction norm.” (Pigliucci et al., 2006)
Meaning, that if a population only experiences the new environmental condition, plasticity will be lost and lead to the evolution of a canalized (fixed) phenotype; as opposed to when the population is continually exposed to both, the old and the new environment, selection will favor the evolution of a reaction norm that is appropriately plastic. In this sense, there exists a clear hierarchy, where phenotypic plasticity should be regarded as a developmental process that can be the target of natural selection, which can lead to the genetic assimilation of that specific trait under certain conditions. This is one route by which plasticity might impact evolutionary innovation and diversification of a species. Phenotypic plasticity and genetic assimilation have been shown to occur in vitro during experimental evolution [251-254]. These traits can be the result of: 1) Single nucleotide polymorphisms in a specific gene [255]; 2) Different mutations that lead to similar phenotypes; 3) Epistatic interaction, such that the expression of a mutation that produces the trait requires one or more preceding mutations [256, 257]. In terms of plasticity, reversion is an important aspect to consider. Reaction norms can be reversed when the inducing condition is removed or the antagonist of that condition is triggered. Traits expressed at the behavioral, biochemical, physiological or developmental levels are significantly different in the degree of reversibility, where biochemical and physiological responses can be reversed over short time scales, whereas developmental plasticity tends to require longer time frames. In this sense, pleiotropy is another important aspect worth consideration.

Pleiotropy is the phenomenon of multiple effects of a single gene or allele on two or more traits. Therefore, a mutation in a pleiotropic gene can affect a myriad of organismal phenotypes, where a change in one trait may either compromise or supplement other traits affected by the same genes [258-260]. Pleiotropy is an important mechanism in social organisms, where underlying molecular mechanisms dictate behavior. Genes involved in regulation and control of secondary messenger c-di-GMP, quorum sensing apparatus, and the two-component signaling systems are examples of pleiotropic genes. These genes are relevant for the adaptation of Pseudomonas aeruginosa in biofilm or non-biofilm conditions where they perform collective behaviors such as pellicle formation or swimming. In manuscript 5, we demonstrate that a population that experiences only the biofilm favoring condition becomes specialized in performing that specific behavior. Regardless of being exposed to new environmental conditions, these ‘specialists’ continue to express high levels of c-di-GMP, a trait that has been assimilated. Since the mutation responsible corresponds to a pleiotropic gene, their performance is decreased in the non-biofilm favoring conditions. However, c-di-GMP expression in P. aeruginosa is controlled by a multitude of genes; therefore, a mutation in one gene can lead to a general change in the reaction norm, but it does not result in an irreversible fixation of that phenotype. The ability of a population to respond to environmental changes is highly subjective, and very much influenced by the life span of the organism. An organism that can live up to 200 years experiences both a longer duration and greater variety of environmental fluctuations versus an organism whose life span does not extend beyond one day. Therefore, an organism cannot always predict the forthcoming environmental changes during its lifespan. So, what is another mechanism that can influence an organism’s ability to adapt to varying conditions?
Bet-hedging is an adaptive strategy in which phenotypic variation is generated stochastically within an isogenic population [261]. Much like an ‘insurance policy’ individuals stochastically develop a phenotype of reduced fitness that may be better suited for a future environment. In doing so, the isogenic population minimizes the temporal variance of surviving offspring and maximizes the mean fitness across varying environments. Even though bet-hedging is confined to isogenic populations, much like phenotypic plasticity, this strategy differs from other survival strategies in the sense that it is an adaptation to variability itself (unpredictable change) and not a specific environmental condition [261, 262]. Phenotypic plasticity relies on the fact that an organism is able to detect environmental cues via sensory circuits and respond accordingly (i.e. responsive gene regulation). Plasticity is a viable option when the environmental variation is in the range of the previously encountered environments and when fluctuations are frequent. However, in the case of infrequent environmental fluctuations, stochastic switching can be advantageous over responsive switching [263]. This is simply because the environmental fluctuations determine the costs and benefits of maintaining the sensory system [264]. Therefore, bet-hedging can prove to be successful when environmental changes are unpredictable and maladaptation bears a severe cost in future environments [262, 263, 265].

In this sense, we dismiss bet-hedging as the main driver for our findings in Manuscript 5. During the ‘alternating’ evolution experiments, Pseudomonas aeruginosa was propagated continuously in either swimming or biofilm favoring conditions. Consequently, under this selection regime, the cells evolved in two directions: they either became ‘generalists’ that were able to switch between the two phenotypes but not maximally fit for either, or specialists with maximized fitness for either swimming or pellicle formation. These phenotypes were in response to the two environmental conditions that were frequently encountered. In addition, we find these behaviors to be mediated via systematic gene regulations in response to the environment and not a stochastic strategy. Therefore, phenotypic plasticity is a more accurate explanation for the strategy observed in Manuscript 5. So how exactly does P. aeruginosa regulate its responsive phenotypic switching?

THE SECONDARY MESSENGER C-DI-GMP

The intracellular secondary messenger bis-(3’-5’)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a ubiquitous biochemical network hub that directs social behavior in bacteria. It is at the core of bacterial signaling pathways, where it serves as a single integrated output for multiple sensory inputs. The advantage of second messenger systems is that their output is modulated by enzymatic synthesis and degradation, making them a rapid mechanism for regulating bacterial processes. C-di-GMP is synthesized from two molecules of guanosine-5’-triphosphate (GTP) by diguanylate cyclases (DGCs) that contain the GGDEF domain [266], and degraded via hydrolysis by phosphodiesterases (PDEs) that contain the either the EAL or HD-GYP domain [267, 268]. The c-di-GMP network sensors (DGCs and PDEs) sense internal or external signals and translate them into c-di-GMP levels, thus modulating the amount of c-di-GMP that specifically binds to downstream effector molecules that cause an alteration in cell physiology and behavior (Fig. 11) [269]. The c-di-GMP responders (including c-di-GMP effectors such as
PilZ, FleQ, and VspT) have motifs (sensors/receptors) that enable them to bind/interact with c-di-GMP and thus directly mediate functions that are a result of the local level of c-di-GMP. There are also non-catalytic (degenerate) EAL, HD-GYP, and GGDEF type domains that are able to bind c-di-GMP and do not participate in the degradation or synthesis of the secondary messenger [270, 271]. This system, although complex, is involved in the regulation and control of biofilm formation [272, 273], proteinaceous appendages (e.g. flagella, pili, adhesins) [274], motility [275], virulence [276, 277], the cell cycle [278], light response [279], and a number of other processes [280].

**Figure 11.** At the cellular level, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is controlled by diguanylate cyclases that carry GGDEF domains (red) and specific phosphodiesterases that carry EAL or HD-GYP domains (blue). C-di-GMP can reduce motility by downregulating flagellar expression or assembly or interfering with flagellar motor function. Low c-di-GMP levels are required for the expression of acute virulence genes. High c-di-GMP levels stimulate various biofilm-associated functions, such as the formation of fimbriae and other adhesins and various matrix exopolysaccharides. The precisely timed and localized action of c-di-GMP is a key step in cell cycle progression. (Reproduced and adapted from Hengge, R. (2009). Principles of c-di-GMP signaling in bacteria. Nature Reviews Microbiology 7, 263–273.)

Perhaps the involvement of this secondary messenger in such a wide variety of functions on a transcriptional, translational, and post-translational level is partly due to the fact that there is a multitude of amino acid motifs and RNA riboswitches that are able to function as sensors for c-di-GMP [281]. For example: domains such as PAS, HAMP, and REC found in proteins containing GGDEF or EAL have been linked to the sensing of redox potential, light, voltage, oxygen, nutrients, osmolarity, antibiotics, homoserine lactones, and a number of other signals [269]; and the widespread PilZ, FleQ, and VspT domains can regulate the activity of enzymes involved in cellulose synthesis, flagellar machinery, surface attachment, the production of alginate and other extracellular polysaccharides [269, 273, 282-286].
Manuscript 5 highlights how varying levels of c-di-GMP influence the motile-to-sessile lifestyle transitions (responsive switching) in *Pseudomonas aeruginosa*, where low levels of c-di-GMP were linked to motility (swimming), whereas high levels were linked to the sessile (pellicle) lifestyle [272]. C-di-GMP modulates functions linked to this change by regulating flagellar motility, adhesion, and synthesis of extracellular polysaccharides. We found that all the isolates from the ‘alternating’ selection regime harbored a mutation in the flagellar gene *fleN* [Manuscript 5]. FleN is known to reduce the activity of FleQ, a protein that induces the expression of flagella biosynthesis genes, thereby regulating the number of flagella in *P. aeruginosa* [287]. Mutation in the *fleN* gene causes the once monoflagellated bacteria to become multiflagellated and confers a growth rate-independent increase in their ability to swim [Manuscript 5]. Our observations are in line with previous studies that demonstrate that mutations in the *fleN* gene can cause increased motility [255], where the mutants become multiflagellated [255, 287]. It is interesting to point out that a mutation in *fleN* was the first mutation to occur in all the isolates in the alternating selection regime and not just the swimming specialists, which suggests that it could have been assimilated in the population. However, mutations in this gene did not result in a locked phenotype, where the ability to regulate c-di-GMP levels allowed strains with the *fleN* mutation to regulate both swimming motility and biofilm formation. Mutations in the Wsp chemosensory system were the primary mechanism for this. The Wsp system consists of seven chemotaxis proteins WspA-F, and a novel response regulator WspR [288]. WspA is a membrane bound methyl-accepting chemoreceptor that responds to as-yet unidentified signal(s), whereas WspC is a methyltransferase and WspF is a methylesterase that regulate the activity of WspA by altering its methylation state (Fig. 12). The hypermethylated WspA activates the diguanylate cyclase WspR by phosphorylation, which then synthesizes c-di-GMP, thereby inducing the biofilm state. Therefore, the Wsp system is important for mediating the shift between the sessile (biofilm) state versus the planktonic (motile) state. The findings in Manuscript 5 indicate that the optimization of one’s ability to respond to environmental changes through such secondary messenger systems is a unique adaptational shortcut to maximizing one’s niche and success when faced with the prospect of migration/relocation and a shift from a global to local competition.

**Figure 12.** Predicted organization of the Wsp signaling system. The methyl-accepting chemotaxis protein homolog WspA detects the signal, which is transferred to WspR by the autophosphorylating activity of WspE. WspR has increased DGC activity, leading to c-di-GMP formation. The methylation state of WspA, determined by opposing activities of the methyltransferase homolog WspC and the methylesterase homolog WspF, is thought to be involved in adaptation to the signal. The dark-blue arrows indicate the location of the HAMP domain and the signal domain of WspA. (Reproduced and adapted from Karatan, E. and Watnick, P. (2009). Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms. Microbiology and Molecular Biology Reviews 73, 310–347.)
**CONCLUSION & FUTURE DIRECTIONS**

*Pseudomonas aeruginosa* is a ubiquitous, versatile, and adaptable gram-negative bacterium colonizing and thriving in diverse ecological niches. In comparison to the knowledge available on its two opposing lifestyles, planktonic and biofilm, we know very little about the shift between the two and the adaptive strategies involved therein. Its broad number of niches emphasizes *P. aeruginosa*'s ability to sense environmental cues and respond accordingly. Evolution experiments and genome sequencing revealed mutational adaptions of strains led to a higher level of responsive phenotypic switching. We find that the ability to shift between two opposing phenotypes such as the planktonic and biofilm state by altering levels of secondary messengers can be a relatively inexpensive way of responding to environmental changes and obtaining plasticity. In addition, experimental conditions simulating the concepts of migration and resettlement shed light on how global versus local competition affects the evolution of an ancestral lineage and the contribution of responsive switching towards fitness. We address this key concept by demonstrating that migration and local versus global competition are key factors that hinder intrusion by specialists.

Delving into the two dominant lifestyles adopted by *P. aeruginosa*, brings forth questions regarding the mutational adaptations and responsive phenotypic switching in its most well-known niche – a live host. This opportunistic pathogen is one of the most common causes of nosocomial infections and has garnered a considerable amount of attention for causing high mortality rates in patients suffering from burn wounds and cystic fibrosis. The former typically involves acute infections with rapid spread and sepsis, whereas the latter involves chronic lung infections harboring biofilms that can last for months or years. We used a murine model of burn infection to ascertain the bacterial fitness and virulence consequences associated with responsive phenotypic switching capabilities of some of the isolates genome sequenced in Manuscript 5. Using this *in vivo* model, we lay the groundwork for our future study. We find that the ancestral wild-type strain was most virulent, causing rapid sepsis and mortality among injured mice, followed by swimming specialists [Manuscript 5 Fig. S3]. In sharp contrast, mice infected with the biofilm specialists did not show any signs of morbidity and mortality. Interestingly, the rates of mortality for mice infected with generalists fell between the rates for those of the swimming and biofilm specialists and were similar to those of mice infected with a mixed population of swimming and biofilm specialists. In addition, we also assessed the bacterial load for the burn wound, region distal to the burn wound, and the spleens. Even though all mice were infected with the same number of bacteria, the group infected with the biofilm specialists had a lower bacterial load in their spleens in comparison to all other groups [Manuscript 5 Fig. S4 and S5]. This suggests a tradeoff between biofilm formation and virulence/fitness during infections in regards to systemic spread. However, if swimming specialists were more fit than biofilm specialists during acute burn infections, one would expect the opposite to be true for chronic infections. Since c-di-GMP regulates the formation and dissolution of biofilms by affecting the synthesis and activities of various cell surface components that function as PAMPs (e.g. flagella, pili, adhesins, and nucleic acids), it has the potential to regulate transitions from acute to chronic infection, and vice versa. However, in the expanding knowledge of c-di-GMP signaling, the significance of this system during infection is often met with skepticism. We aim to address this topic in our future studies.


89. Davies NB, Quinn D. Cuckoos, Cowbirds and Other Cheats: Bloomsbury Publishing; 2010.


60


Manuscript 1
A post-planktonic era of in vitro infectious models: issues and changes addressed by a clinically relevant wound like media

Urvish Trivedia, Jonas S. Madsen, Kendra P. Rumbaugh, Randall D. Wolcott, Mette Burmølle and Søren J. Sørensen

ABSTRACT
Medical science is pitted against an ever-increasing rise in antibiotic tolerant microorganisms. Concurrently, during the past decade, biofilms have garnered much attention within research and clinical practice. Although the significance of clinical biofilms is becoming very apparent, current methods for diagnostics and direction of therapy plans in many hospitals do not reflect this knowledge; with many of the present tools proving to be inadequate for accurately mimicking the biofilm phenomenon. Based on current findings, we address some of the fundamental issues overlooked by clinical labs: the paradigm shifts that need to occur in assessing chronic wounds; better simulation of physiological conditions in vitro; and the importance of incorporating polymicrobial populations into biofilm models. In addition, this review considers using a biofilm relevant in vitro model for cultivating and determining the antibiotic tolerance and susceptibility of microorganisms associated with chronic wounds. This model presents itself as a highly rapid and functional tool that can be utilized by hospitals in an aim to improve bedside treatments.

ARTICLE HISTORY
Received 22 July 2016
Accepted 20 October 2016
Published online 18 November 2016

KEYWORDS
Chronic infections; biofilms; polymicrobial; antibiotic tolerance; wound care

Introduction
Hospital clinical labs follow the standard antimicrobial susceptibility testing (AST) guidelines when determining appropriate antibiotics to be administered and their minimal inhibitory concentrations (MICs) (CLSI, 2014). However, obtaining MICs for microorganisms originating from chronic infections by using standard AST assays more often than not results in false positives (Costerton et al., 1995). This is largely because the planktonic cell paradigm is an inaccurate representation of how bacteria behave in chronic infections. A chronic wound is an example of a biofilm-related chronic infection (Hoiby et al., 2015). One of the greatest factors that influences the ability of a chronic wound to be resolved is the presence of biofilm (Watters et al., 2013). The Center for Disease Control and Prevention estimates that biofilm phenotype bacteria cause 65% of all human infectious diseases and the National Institutes of Health notes that this is closer to 80% (NIH, 1997). Biofilms are communities of microbial cells attached to a surface or each other and encased in a polymeric matrix. First ushered into medicine by Costerton in 1985, the significance of biofilm-related infections is now widely accepted (Costerton et al., 1978). Despite the overwhelming number of studies discussing biofilms as the predominant microbial lifestyle in nature as well as chronic infections (Costerton et al., 1995; Davey & O'Toole, 2000; Donlan, 2002; Donlan & Costerton, 2002; Parsek & Fuqua, 2004; Watnick & Kolter, 2000; Webb et al., 2003), planktonic liquid cultures remain the model for many microbiology studies today. This is especially problematic when bacteria are isolated from a biofilm-related infection, such as chronic wounds (Bjarnsholt, 2013; James et al., 2008), and are then cultivated and studied using traditional methods that return the bacteria to their planktonic state (Costerton et al., 1999). The planktonic phenotype is vastly different from the biofilm phenotype (Stoodley et al., 2002). Planktonic cultures lack structure, cells are not adhered, or surrounded by a polymeric matrix, and the transcriptomic and proteomic profiles of planktonic versus biofilm cells differ dramatically (Sauer et al., 2002). Most importantly, planktonic cells are typically much more susceptible to treatments such as antibiotics than biofilm cells as will be discussed further.

CONTACT Prof. Søren J. Sørensen sjs@bio.ku.dk Department of Biology, University of Copenhagen, Universitetsparken 15 Bldg 1, Copenhagen, 2100, Denmark.
© 2016 Informa UK Limited, trading as Taylor & Francis Group
(Stewart & Costerton, 2001). Therefore, standard AST assays provide excellent in vitro results, when tested on planktonic cells, but translate poorly in vivo, where the biofilm phenotype predominates (Sepandj et al., 2004). This biased assessment undoubtedly contributes to why many antibiotic therapies prove unsuccessful for bedside treatments. The failure of pharmaceutical industries to deliver useful hit drugs that could be used for treating biofilm-related infections is that majority of the antimicrobials on the market were developed against planktonic cells. So, it should be no surprise that they are rendered ineffective in treating chronic infections. An immediate aim should therefore be to conduct pre-clinical trials using biofilm relevant models. This will refocus the discovery process back onto a bacteria-community level, but also specifically target the biofilm mode of growth.

It has been shown in vitro that bacteria residing in biofilms can be up to 1000 times more tolerant to antibiotic agents than free-floating planktonic bacteria (Mah & O’Toole, 2001). The key term associated with this observation is “tolerance”. Clinical laboratories often take into account antibiotic resistance of organisms when profiling their susceptibilities, but rarely do they account for antibiotic tolerance. Despite the fact the latter phenomenon can lead to the development of antibiotic ineffectiveness, there is a clear distinction between the two. Unlike, conventional antibiotic resistance, which is typically caused by transferable genetic alterations or specific resistance genes that confer protection against antimicrobials, tolerance implies a transient, non-heritable phenotype often associated with the biofilm lifestyle (Trivedi et al., 2014). Both, antibiotic resistance and tolerance facilitate survival of pathogens and are therefore highly relevant in the clinic. It is therefore a significant oversight to only test resistance, as is the case when standard AST assays are conducted using planktonic cultures. It is the purpose of this review to bring a highly relevant in vitro biofilm model to the attention of clinical microbiologists and infectious disease specialists. In light of current advances in microbiology, we argue that improved diagnostics is achievable if future models are developed to incorporate key factors such as the biofilm phenotype, polymicrobial phenomenon and concepts of antibiotic tolerance, as opposed to purely focusing on resistance.

In order for a model to be clinically applicable, it must meet several criteria: (i) closely resemble the environment encountered within chronic wounds (e.g. nutrient availability, pH, etc); (ii) allow for culturing and rapid screening of pathogens found in chronic wounds; (iii) support the polymicrobial growth of key microorganisms present in chronic wounds; (iv) reflect a high level of antimicrobial tolerance as observed in wounds; (v) allow for parallel testing of both bacteria- and biofilm-targeted treatments; (vi) must be commercially and economically viable. We believe that all of these criteria are adequately addressed by the wound-like media (WLM) model that is now actively being utilized in clinical practice for treating chronic wounds and answering basic research questions. It is critical to develop similarly versatile and easily applicable models for non-wound associated infections meeting the aforementioned criteria.

**Current, frequently used in vitro biofilm models**

Development of many low cost in vitro screening methods, such as flow cells, drip flow reactors (DFR), CDC biofilm reactor (CBR) and microtiter plate based screening assays has led to many advancements in basic biofilm research. The Calgary Biofilm Device (Figure 1) is actively used for the rapid screening of biofilm susceptibilities to antimicrobial agents (Ceri et al., 1999; Das et al., 1998; Harrison et al., 2010; Jakobsen et al., 2012; Pitts et al., 2003). Its two-part components consist of a 96-well plate and a lid with pegs, which extend into each well filled with static liquid media. Each peg is engineered with a break point above the liquid-air-surface interface that allows it to be removed from the lid with needle nose pliers (Harrison et al., 2010). In this model, both the pegs and the sides of the wells serve as biofilm growth surfaces. Bacterial cells can be obtained from the pegs, for viable cell counting, either via sonication or mechanical removal. However, this step is laborious and often introduces large variability among replicates. This microtiter plate based screening is, therefore, typically used for quantifying the overall biofilm biomass through popular staining methods (e.g. Crystal Violet and Syto9). Crystal violet is a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li et al., 2003). However, Crystal Violet is poorly suited to evaluate killing of biofilm cells for viability assays, since it stains the matrix and does not differentiate between...
the case when standard AST assays are conducted using and are therefore highly relevant in the clinic. It is there-
tection against antimicrobials, tolerance implies a transi-
tions or specific resistance genes that confer pro-
ter phenomenon can lead to the development of anti-
account for antibiotic tolerance. Despite the fact the lat-
when profiling their susceptibilities, but rarely do they

Unlike conventional antibiotic resist-
biotic ineffectiveness, there is a clear distinction

An immediate aim should therefore be to conduct pre-

Development of many low cost models, such as flow cells, drip flow reactors (DFR), CDC biofilm reactor (CBR) and microtiter plate based screen-
ods, such as flow cells, drip flow reactors (DFR), CDC

An immediate aim should therefore be to conduct pre-

The synthetic cystic fibrosis sputum media (SCFM) and artificial sputum media (ASM) are elegant in vitro models for mimicking dental microbes and are not suitable for modeling other types of chronic infections, including wound research (Exterkate et al., 2010; Foster & Kolenbrander, 2004; Kara et al., 2006; Kreth et al., 2005; Peyyala et al., 2011; Xie et al., 2008). The commonality and general bias between these methods is that they all represent biofilms attached to abiotic surfaces, thus accounting only for the role of bacterium-derived matrix in antimicrobial tolerance.

The Calgary microtiter plate is perhaps the most widely used model system used to generate biofilms, most of the disease-related systems have been focused on dental microbes and are not suitable for modeling other types of chronic infections, including wound research (Exterkate et al., 2010; Foster & Kolenbrander, 2004; Kara et al., 2006; Kreth et al., 2005; Peyyala et al., 2011; Xie et al., 2008). The commonality and general bias between these methods is that they all represent biofilms attached to abiotic surfaces, thus accounting only for the role of bacterium-derived matrix in antimicrobial tolerance.

The synthetic cystic fibrosis sputum media (SCFM) and artificial sputum media (ASM) are elegant in vitro models for mimicking dental microbes and are not suitable for modeling other types of chronic infections, including wound research (Exterkate et al., 2010; Foster & Kolenbrander, 2004; Kara et al., 2006; Kreth et al., 2005; Peyyala et al., 2011; Xie et al., 2008). The commonality and general bias between these methods is that they all represent biofilms attached to abiotic surfaces, thus accounting only for the role of bacterium-derived matrix in antimicrobial tolerance.

The Calgary microtiter plate is perhaps the most widely used model system used to generate biofilms, most of the disease-related systems have been focused on dental microbes and are not suitable for modeling other types of chronic infections, including wound research (Exterkate et al., 2010; Foster & Kolenbrander, 2004; Kara et al., 2006; Kreth et al., 2005; Peyyala et al., 2011; Xie et al., 2008). The commonality and general bias between these methods is that they all represent biofilms attached to abiotic surfaces, thus accounting only for the role of bacterium-derived matrix in antimicrobial tolerance.

The synthetic cystic fibrosis sputum media (SCFM) and artificial sputum media (ASM) are elegant in vitro models for mimicking dental microbes and are not suitable for modeling other types of chronic infections, including wound research (Exterkate et al., 2010; Foster & Kolenbrander, 2004; Kara et al., 2006; Kreth et al., 2005; Peyyala et al., 2011; Xie et al., 2008). The commonality and general bias between these methods is that they all represent biofilms attached to abiotic surfaces, thus accounting only for the role of bacterium-derived matrix in antimicrobial tolerance. The Calgary microtiter plate is perhaps the most widely used model system used to generate biofilms, most of the disease-related systems have been focused on dental microbes and are not suitable for modeling other types of chronic infections, including wound research (Exterkate et al., 2010; Foster & Kolenbrander, 2004; Kara et al., 2006; Kreth et al., 2005; Peyyala et al., 2011; Xie et al., 2008). The commonality and general bias between these methods is that they all represent biofilms attached to abiotic surfaces, thus accounting only for the role of bacterium-derived matrix in antimicrobial tolerance.

Unlike the other in vitro biofilm models and systems, the WLM is extremely easy and straightforward to prepare. It does not require the preparation of complex buffers, amino acid stocks, tubing, peristaltic pumps and large volumes of media. It simply requires the user to mix three components, as specified, to obtain final concentrations for working volumes. After which it can be inoculated to generate a mature biofilm within 24 h. Prior to preparation, all three components are stored separately to maintain longevity and stabilization. So what exactly does the WLM consist of?

**Wound-like media**

Sun et al. (2008) have developed a simple yet elegant in vitro biofilm model (Figure 2) that has proved to be a functional system for testing the efficacy of antimicrobials, and could easily be adapted to test other therapeutics or host factors. Made from commercially available components, WLM represents the conditions of human wounds and contains physiological concentrations of blood components. Many studies note that the major host factors encountered within a wound bed are damaged tissue, red blood cells and plasma (Attinger & Wolcott, 2012; Wolcott & Rhoads, 2008). Relevantly, WLM is composed of just that: 45% chopped meat-base media (Bohlon broth), 50% bovine plasma and 5% freeze-thawed laked-horse red blood cells (RBC) (Sun et al., 2008). The bovine plasma component of the WLM model contains (7.9%) protein, and the main proteins are the albumins (3.3%), immunoglobulins, α- and β-globulins (4.2%), and fibrinogen (0.4%) (Howell & Lawrie, 1983), all of which are present in chronic wounds. The addition of laked-blood and the chopped meat-based media allows for better simulating the physiological iron, carbohydrate and host protein levels.

**Figure 2.** Visual comparison of an actual debridement sample taken from chronic wound biofilm with the wound-like media. This image compares a typical chronic wound biofilm on the left and the wound-like media on the right showing the similarity in texture and consistency between the in vivo biofilms and the in vitro model (Sun et al., 2008).
all of which should be considered when profiling antibiotic tolerance and susceptibilities in chronic wounds.

Bacterial biofilm formation can be studied in the WLM model either by (i) introducing a biotic or abiotic surface whereon the bacteria can attach and form a biofilm, (ii) letting the blood-based medium coagulate naturally by excluding the anticoagulants in the bovine plasma or (iii) introducing a coagulase positive species (such as coagulase positive Staphylococcus aureus) to let the liquid medium coagulate. The latter is due to the ability of S. aureus to activate the coagulation cascade. S. aureus secretes the cofactor staphylocoagulase, which activates prothrombin in a non-proteolytic manner, resulting in a complex called staphylothrombin, which converts soluble fibrinogen to insoluble fibrin strands (Zajdel et al., 1975). It should be noted that the WLM does not coagulate upon addition of an anticoagulant, after which coagulase negative species will not coagulate the blood components, because they are unable to activate the coagulation cascade. Therefore, in this model, the coagulated plasma and host-derived matrix serve as a scaffold to which bacteria can adhere to and can form a biofilm. We propose that this model closely reflects the in vivo wound environment and would also help clinicians and researchers better understand the role of host-derived matrix components in wound infections. In the following section, we describe key issues often overlooked when dealing with chronic wounds and how models such as the WLM can be applied in the clinic as well as for biofilm research.

Microbial identification in chronic wounds

After admission into hospitals, patients suffering from chronic infections typically receive initial clinical assessment and their wounds are examined. Following their primary evaluation, a microbiology report is requested by the attending physician to identify the organisms present and begin course of treatment. Although many studies have demonstrated the superiority of molecular diagnostic techniques (Kessler et al., 2006; Dowd et al., 2008; Pellizzer et al., 2001), it is interesting to point out that many hospitals still rely on Koch’s postulates (1884), one of which states that the microorganism must be isolated and grown in pure culture form. Thus, following a surface swab of the wound, bacteria are enumerated and profiled after having been grown in liquid broth. Relying purely on this outdated approach results in clinical labs being able to culture only viable pathogens from wounds (typically nonfastidious, aerotolerant microorganisms), giving practitioners a misrepresented view of the actual microbial population and an incorrect antibiotic susceptibility profile.

Epidemiological studies report Staphylococcus aureus, coagulase-negative staphylococci, Streptococcus spp., Enterococcus spp., Corynebacterium spp., Enterobacteriaceae and Pseudomonas aeruginosa to be the most prevalent aerobes isolated from diabetic chronic wounds (Lipsky, 2008). Whereas, Gram positive cocci, Prevotella spp., Porphyromonas spp. and Bacteroides fragilis are the most predominant anaerobes (Lipsky, 2008). However, research groups utilizing DNA sequencing have revealed that up to 90% of bacteria in wounds are facultative or obligate anaerobes (Dowd et al., 2008; Price et al., 2009). Since anaerobes are typically sensitive to oxygen, special precautions must be taken when obtaining and transporting samples. This often requires physicians to specially order tests to screen for anaerobes. Even then, an anaerobic organism does not propagate in a pure shaker culture exposed to oxygen, and are rarely reported in the initial microbiology screenings (Lipsky, 2008).

Technological advances have provided medical science with many high-throughput sequencing systems. Undoubtedly, molecular diagnostic methods have proven to be much more sensitive and accurate for the rapid identification of pathogens compared to cultivation-dependent methods (Casman, 2004). Techniques such as polymerase chain reaction (PCR), multilocus sequence typing and 16S rRNA gene sequencing have proven to be effective in identifying both gram-positive and gram-negative, as well as both aerobic and anaerobic organisms in various types of wounds. However, these detection methods do not discriminate between live and dead bacterial DNA. A way to address this issue is to use PMA-seq, a DNA sequencing method that uses propidium monoazide (PMA) to differentiate between living and dead microbes. PMA is unable to pass through intact cell membranes; therefore, it only binds to DNA of compromised cells (Nocker et al., 2007, 2009; Vaishampayan et al., 2013). Thus, PCR amplification of DNA from a PMA-treated sample amplifies DNA only from living cells (Kim et al., 2013; Oketic et al., 2015; Yasunaga et al., 2013). Another potentially useful diagnostic technology is the oligonucleotide array for detecting various genes, including those encoding resistance, toxins and distinguishing specific species (Heller, 2002). Even though high throughput sequencing of 16S rRNA gene amplicons makes it possible to identify bacterial species in mixed samples, it does not provide insight into how the inter-species interactions enhance their tolerance towards antibiotics. Metagenomic methods, examining all genes present, does not have the limitations of amplicons and therefore identifies microorganisms to the strain level and just as importantly identifies (and quantitates) mobile
genetic elements (resistance and virulence factors). This limitation renders these methods useful in identifying bacterial species and only their antibiotic resistance genes, but fails to assess their biofilm-related-tolerance in chronic wounds.

Using the WLM, Sun et al. (2009) were able to generate a polymicrobial biofilm including several key anaerobic bacteria within 24 h. All strains chosen for the study had previously been associated with chronic wounds: Peptoniphilus ivornii, Pepto-streptococcus anaero-bius, Anaerococcus lactolyticus, Finegoldia magna and Clostridium perfringens. Even though many of them are known to cause gas gangrene, brain, liver, breast and lung abscesses, all of them are associated with infectious biofilms and enhanced chronicity of surgical site infections, and diabetic foot and pressure ulcers. After inoculation, they monitored the population dynamics within the WLM for 48 h using quantitative PCR. Not only were the anaerobes able to successfully integrate themselves into the matrix, but also their bacterial population thrived within the biofilm. Scanning electron microscopy revealed that all of the inoculated strains were present in close proximity within the differing thickness of the biofilm (Figure 3). This type of coexistence of aerobes and anaerobes is not possible in standard liquid cultures due to the lack of a spatially structured community. In the WLM, the aerobic species consume the diffused oxygen, creating degrees of anaerobic conditions within the community. As the biofilm grows in thickness, the lower parts of the community become deprived of oxygen, thus creating conditions in which the anaerobes can survive and proliferate. It is speculated that this dynamic in vivo allows the anaerobes to interfere with phagocytosis of not only themselves, but also the aerobes present within mixed species infections (Ingham et al., 1977). These environmental micro-alterations such as oxygen gradients, pH differences and community structuring involves a level of dependency on each other; creating a microenvironmental heterogeneity, which facilitates growth of diverse bacteria (Pande et al., 2015). However, when it comes to culturing, some of these aerobes present complications of their own.

**Coculturing and maintaining key microbial populations**

*P. aeruginosa* and *S. aureus* are the two most notorious organisms associated with chronic wounds (Bessa et al., 2013; Fazli et al., 2009; Gjodsbol et al., 2006; Korber et al., 2010; Lipsky, 2008; Trivedi et al., 2014) and antimicrobial resistance (Abdulrazak et al., 2005; Bansal et al., 2008; Ramakant et al., 2011). Many studies note that infections harboring both, *S. aureus* and *P. aeruginosa* are often more recalcitrant and/or result in poorer prognosis than mono-infections (Hendricks et al., 2001; Pastar et al., 2013; Rosenbluth et al., 2004). Although they are frequently found together in human infections, *P. aeruginosa* kills *S. aureus* when the two are cocultured in most standard broth media (DeLeon et al., 2014; Palmer et al., 2005, 2007). There are several factors that are attributed to the killing of *S. aureus* when it is cultured with *P. aeruginosa* in liquid broth. Similar observations were made when the two pathogens were cultured in CF sputum medium, where *P. aeruginosa* induces several distinct staphyloytic factors and preemptively lyses *S. aureus* (Palmer et al., 2005). Specifically, *P. aeruginosa* uses peptidoglycan shed by Gram-positive bacteria as a social cue to produce quinolone signal (PQS)-controlled factors elastase and pyocyanin, thereby enhancing its virulence against *S. aureus* (Korgaonkar et al., 2013). Likewise, the presence of *P. aeruginosa* induces expression of *S. aureus* virulence factors Panton-Valentine leukocidin and *S*-hemolysin (Pastar et al., 2013). Although the two species can co-exist together in wounds, many of their extracellular factors not only possess lytic activity against bacteria, but also eukaryotic cells. Therefore, it is crucial to take into account the antimicrobials and toxins that not only modify the composition of the community but also enhance host killing in polymicrobial infections.
wound infections. The fact that *P. aeruginosa* and *S. aureus* can only co-exist in relevant biofilm models that mimic wound conditions illustrates how important it is to choose the correct assay when researching bacterial biofilms.

By utilizing the WLM model, Dalton et al. (2011) were able to study how pathogens interact in a wound environment. Four of the aerobic and anaerobic species most commonly isolated from human wounds were chosen for the aforementioned study: *S. aureus*, *P. aeruginosa*, *Enterococcus faecalis* and *F. magna*. During their initial assessment, using planktonic cultures, they were unable to establish a polymicrobial murine infection of some of these strains. However, they were able to grow a polymicrobial biofilm in the WLM for two to four days (Dalton et al., 2011). In an aim to understand how polymicrobial biofilms affect the host immune system and healing, they utilized a clinically relevant murine chronic wound model (Watters et al., 2013). Biofilms were grown for four days, and then aseptically removed and sectioned. Biofilm sections were then transplanted onto the surgical excision wounds of mice (Dalton et al., 2011). PCR analysis revealed that the population distribution of the species in the starting WLM biofilms did not differ substantially to that of any of the time points examined in vivo (Figure 4) (Dalton et al., 2011). These data indicate that the WLM can be used to establish a polymicrobial biofilm that can be transplanted and maintained even in an *in vivo* clinical model.

It should be noted that sampling techniques and biofilm-facilitated tolerance also play a major role in wound assessments. Numerous studies have demonstrated the superiority of obtaining deep tissue biopsies for culturing (Pellizzer et al., 2001; Zajdel et al., 1975). This is because superficial swabs of wounds often provide an inaccurate representation of the microbial population in wounds. Deep tissue biopsy samples can be used for molecular diagnostics to accurately identify specific species and their resistance genes. In addition, biopsy samples can also be used to inoculate the WLM in order to assess the biofilm and the tolerance of polymicrobial communities in chronic wounds. This is an important aspect to consider when studying and treating chronic infections, where in many cases it is likely that key pathogens will encounter and co-exist with other bacterial communities/partners. Identifying the type and number of bacteria present should be the first aim in assessing chronic wounds. Thus, where planktonic cultures and other models are failing short, the WLM model bears great potential as a screening tool for polymicrobial infections.

**Importance of antimicrobial tolerance in polymicrobial communities**

Due to the technical challenges of co-cultivating multiple species of bacteria, the critical factor that is almost always overlooked is the effects of polymicrobial interactions on antibiotic susceptibility. Utilization of the "single organism single disease" theory often results in clinicians administering sub-optimal doses of antibiotics based on monoculture MIC profiles. The ramifications of practicing this ideology end with many of the co-inhabiting strains developing resistance or becoming more virulent over the course of the infection. For example, some studies have noted this phenomenon in treating polymicrobial infections with *S. aureus* and *P. aeruginosa*. In a controlled study, treatments with only anti-staphylococcal antibiotics subsequently resulted in enhanced colonization of *P. aeruginosa* and its transformation to a more virulent mucoid phenotype in cystic fibrosis patients (Marks et al., 1990). Another study has demonstrated that both *S. aureus* and *P. aeruginosa* display higher tolerance to some antibiotics when co-cultured together (DeLeon et al., 2014).

Social interactions are key driving forces for shaping community composition and spatial organization within polymicrobial infections. Many bacteria are able to produce various signals and enzymes to communicate with
each other on both intra- and inter-species level. In turn, some of these extracellular factors can be considered “public goods” since they provide indirect benefits for the community as a whole. For example, many of the polymicrobial infections harboring *P. aeruginosa* often go unresolved when treated with streptomycin, gentamicin and beta-lactams. This is because *P. aeruginosa* produces several aminoglycoside-modifying enzymes and beta-lactamase, which can then in turn protect many of the co-inhabiting strains (Connell et al., 2013; Poole, 2005). In addition, the growth and metabolic stratification within biofilms has an adverse effect on the efficacies of many antibiotics that only act on proliferating cells. Aerobic bacteria residing on the surface experience a higher concentration of oxygen in comparison to those located in the center and lower parts of a biofilm, thus many bacteria adopt a sessile and dormant lifestyle (Costerton et al., 1995). For example, Hoffman et al. (2006) demonstrated that *P. aeruginosa* exoprotein 4-hydroxy-2-heptylquinoline-N-oxide could protect *S. aureus* from killing by the aminoglycoside tobramycin in *S. aureus*-*P. aeruginosa* cocultures by suppressing *S. aureus* respiration. This shift from aerobic respiration to fermentation can shunt certain cells from an active/proliferative lifestyle to a dormant state, allowing persister cells to survive and exist within infectious polymicrobial biofilms. Persisters are dormant cells that arise stochastically in some microbial populations (Lewis, 2007). When left untreated, these cells can re-establish a biofilm within the host, often resulting in a recalcitrant infection. These are all examples of indivisible emergent properties present within polymicrobial infections, which are otherwise absent when assessing monocultures.

Another emergent property observed within polymicrobial communities is enhanced biofilm formation. Wounds harboring multiple species of bacteria usually present with greater amounts of biofilm than monospecies infections (Hendricks et al., 2001; Pastar et al., 2013; Rosenbluth et al., 2004). These pathogen interactions not only prove problematic for the host when occurring between bacterial or fungal species, but also when occurring between the two organisms. This is because intra- and inter-specific interactions lead to either synergy or competition among the strains present, both of which can lead to enhanced biofilm formation and production of extracellular factors (Connell et al., 2013; Korgońkar et al., 2013; Ren et al., 2014; Rumbaugh et al., 2012; Stacy et al., 2014). Harriott & Noverr (2009) demonstrated that *S. aureus* forms substantial polymicrobial biofilm in the presence of *C. albicans* in comparison to a monomicrobial biofilm; where *C. albicans* serves as the underlying scaffolding, allowing *S. aureus* to form microcolonies on the biofilm surface (Harriott & Noverr, 2009). Furthermore, *S. aureus* enhanced tolerance to vancomycin within polymicrobial biofilms was in part mediated by *S. aureus* becoming coated in the matrix secreted by *C. albicans* (Harriott & Noverr, 2009). In addition, physical interactions with candidal hyphae may provide *S. aureus* with an invasion strategy through host epithelial layers and upregulate staphylococcal proteins that confer resistance to host-derived oxidative stressors (Peters et al., 2010). These interactions are also observed in clinical settings where both species often form polymicrobial biofilms on dentures, prostheses and intracardiac catheters removed from patients (Baena-Monroy et al., 2005; Costerton et al., 1985; Tawara et al., 1996). Therefore, the significance of understanding the role of underlying molecular mechanisms of interspecific interactions and the associated emergent properties of mixed species biofilms is crucial in treating infections.

**Role of EPS and host-derived matrix in chronic wounds**

The biofilm matrix can consist of both a combination of bacterial extracellular polymeric substances (EPS) and host-derived matrix (e.g. fibrin and collagen). Undoubtedly, the biofilm matrix can greatly influence the penetration of certain antimicrobials. Generally, the overall net charge of the EPS matrix is negative; thus, negatively charged and neutral antibiotics bear higher penetrability in comparison to those that are positively charged. Recently, Tseng et al. (2013) demonstrated that the positively charged antibiotic tobramycin became sequestered in the biofilm peripheries, while a neutral antibiotic readily penetrated them. Other drug susceptibility studies have demonstrated that fungal cells can modulate the action of antibacterial agents while bacterial cells can affect the activity of antifungal agents (Adam et al., 2002; Jenkinson, 2002). For example, extracellular polymers produced by *Staphylococcus epidermidis* are able to inhibit fluconazole penetration, while the presence of *C. albicans* protects the *S. epidermidis* against vancomycin in mixed fungal-bacterial biofilms (Adam et al., 2002; Jenkinson, 2002). This tolerance is completely dependent on being in the biofilm environment. Thus, once a microbe is removed (e.g. swab culture) and proliferated *in vitro*, subsequent generations of planktonic cells will revert back to their susceptible phenotypes (Trivedi et al., 2014). This misunderstanding often leads to clinical labs generating inaccurate antibiotic susceptibility reports.

Another factor that is overlooked is the presence of high amounts of host-derived matrix in wounds. In
addition to microorganisms, host components, such as fibrin, platelets or immunoglobulins, may be integrated into the biofilm matrix. In the presence of plasma, *S. aureus* is able to form a host derived matrix by “hijacking” the host coagulation cascade, where soluble fibrinogen is converted to insoluble strands of fibrin (Zajdel et al., 1975). The resulting interconnected fibrin network of the extracellular matrix components have been suggested to act as a mechanical barrier that perturb the penetration of antimicrobials. Therefore, therapies directed at disrupting both bacterial- and host-derived matrix should be assessed in treating chronic wounds. However, the type and doses for topical treatments cannot be ascertained using standard liquid cultures that are lacking in both structure and host factors.

The ability to accurately mimic spatial organization of wound biofilms is an important feature considering the discrepancies between *in vitro* biofilm models and *in vivo* biofilms in chronic infections. Many research groups utilizing biofilm systems, such as flow cells, study biofilms growing in mushroom shaped structures formed by *P. aeruginosa*. The mushroom-shaped structures are composed of a stalk and a cap; the stalk is formed by clonal growth, whereas motile bacteria climbing the stalks by help of type IV pili and form the caps of the mushrooms (Bjarnsholt et al., 2013; Klausen et al., 2003). In an attempt to understand this key structural characteristic and behavior of *P. aeruginosa* biofilms, multiple studies have been carried out. Despite academic interests in such phenomenon, these mushroom-like structures have not yet been observed *in vivo* (Bjarnsholt et al., 2013). Many studies report that pathogenic biofilms often grow as non-surface associated aggregates; therefore, it is a common misconception that the biofilm matrix present in wounds is always produced by bacteria (Donlan & Costerton, 2002; Hall-Stoodley et al., 2004; Jensen et al., 2010; Parsek & Singh, 2003). In fact, the *in vivo* biofilm patches observed ranged from 5 to 200 μm in diameter, growing as small aggregates, distributed throughout the infected host tissue (Bjarnsholt et al., 2013). This is a fine example illustrating the discrepancies between the non-relevant *in vitro* biofilm models and *in vivo* biofilms in actual chronic wounds. Surprisingly, there are key structural commonalities between the biofilms generated using the WLM and those found in a chronic wound (Figure 5) (Sun et al., 2008).

The WLM model accounts for the large amount of host-derived matrix present in chronic wounds that can greatly influence the implemented treatment plans. Using the WLM biofilm model, studies have been able to profile the effects of antibiofilm agents such as Triclosan, gallium (III) nitrate, cadexomer iodide, xylitol, erythritol, farnesol, salicylic acid and two prototype Sanguitec gels (Dowd et al., 2009; Kucera et al., 2014; Sun et al., 2008). Unlike antibiotics, many of these agents do not target the bacteria directly, but rather the biofilm matrix. Katsuyama et al. (2005) reported that xylitol and farnesol synergistically inhibited *S. aureus* biofilm formation: xylitol inhibited the formation of the glycocalyx and farnesol dissolved the fibrin fibers. These types of treatments are highly recommended since they directly target the biofilm, shunting the sessile/dormant microorganisms into an active lifestyle. While reconstituting the degraded biofilm, the residual microorganisms become more vulnerable to subsequent treatments of antibiotics and biocides (Wolcott et al., 2010). The WLM model would allow clinicians to develop personalized biofilm-based strategies for patients, for which multiple agents can be used simultaneously or in a step-wise manner.

**Benefits of “biofilm-based wound care”**

Many patients experience the “standard care” practice over the course of their wound care. This includes reperfusion, nutritional support, offloading, compression and management of systemic disease (Wolcott et al., 2010). Although this regimen is widely accepted amongst clinicians, it must be stressed that additional, yet alternate course of treatments are required when dealing with chronic wounds, particularly those harboring biofilms. The Southwest Regional Wound Care Center in Texas, USA is a freestanding, comprehensive center involved in the management of all types of
chronic wounds. Their medical practitioners have adopted a very manageable rubric called "biofilm-based wound care" as part of their clinical practice.

Biofilm-based wound care starts by sampling and characterizing the wound bioburden by molecular methods at first encounter. Additional biopsy samples are used as inoculum for the WLM, which is used to generate biofilm appropriate susceptibility and tolerance reports. The wound is debrided of all slough, devitalized tissue and/or foreign bodies. Next any architecture that favors biofilm formation, such as tunnels and undermining are altered or removed. Following debridement, antimicrobial wound cleanser is applied followed by concurrent application of specific antibiotics, anti-biofilm agents and selective biocides to target the microbes identified by molecular methods (Wolcott, 2015). "Biofilm-based-wound care" management strategies are much more robust than standard antibiotic treatments based on liquid cultures. Designed to suppress and eradicate biofilms, these types of treatments will cover any planktonic phenotype microorganisms present in wounds (Wolcott & Rhoads, 2008). By implementing this system in clinical practice, practitioners have reported a significantly higher healing rate among patients being treated at the Southwest Regional Wound Care Center (Wolcott & Rhoads, 2008). Utilizing the WLM, a biofilm relevant clinical model, has allowed for the testing of new anti-biofilm agents and implementation of better combinational therapies for treating chronic wounds (Attinger & Wolcott, 2012; Sun et al., 2008; Wolcott & Rhoads, 2008). Likewise, other institutions and clinical labs can also better assess current methods, and adjust conventional antibiotic doses to those needed for treating biofilm infections; all in an aim towards curbing the overuse of "gold standard" and "last resort" antibiotics.

There are also large financial incentives and economic benefits to be considered for both patients and institutions when treating chronic wound infections. In a retrospective study involving 404 patients undergoing standard care methods versus "biofilm-based wound care", Wolcott (2015) observed that the supplies per patient was reduced significantly but not proportionately. Second, the number of new patients per month increased because of faster healing rates. Additionally, they saw a 73% reduction in total patient cost for complete wound healing. This sharp decrease was attributed to visits and less complex procedures, and a vast reduction in the use of systemic antibiotics, such as daptomycin, linezolid and tigecycline. The "biofilm-based wound care" regimen for these patients involved regular wound debridement and the use of topical antibiotics, biocides and/or biofilm agents that are much less likely to lead to developing resistance (Wolcott, 2015). With increased success of rapidly healing wounds, they have experienced new and larger volumes of patients seeking "biofilm-based wound care" at their center.

**Concluding remarks**

The WLM addresses many of the shortcomings of standard liquid cultures and agar plates currently used by hospital clinical labs. The WLM provides a very representative nutrient base to that found in chronic wounds. It contains many of the host-derived matrix and components found in wound exudates. Inoculated bacteria have the ability to grow as attached biofilms or non-attached aggregates that experience varying oxygen gradients upon biofilm maturation, enmeshing themselves in and around the host matrix components as observed in vivo chronic wound biofilms. Most importantly, it is a highly relevant clinical model for co-cultivating multiple species to better represent the interactions observed in polymicrobial infections. This allows clinical labs to obtain full and comprehensive tolerance and susceptibility reports on not just the strains present, but also the biofilm community as a whole. As it’s easy to handle and bears great commercial applicability, it should enable clinical labs to simultaneously and rapidly test multiple antimicrobials in parallel.

Medical practitioners rely on accurate laboratory results to direct therapy and support infection control or antibiotic stewardship (Bartlett et al., 2013; Boucher et al., 2009; CDC, 2013; European Centre for Disease and Control, 2014; WHO, 2014; Hoang et al., 2013; Pfeiffer & Beldavs, 2014; Talbot et al., 2006). Therefore, it is imperative that the Clinical and Laboratory Standards Institute (CLSI) begin reevaluating the methods for determining microbial populations and their antimicrobial susceptibilities. The CLSI’s in vitro testing models should incorporate the biofilm phenomenon in successfully treating wound infections. Having clinical labs shift away from traditional AST methods will undoubtedly result in internal costs during the initial transition phase. However, since all of the components required for utilizing and integrating the WLM into practice are already commercially available, the long-term costs would be minimal in comparison to those resulting from inefficient use of antibiotics. False positives, inadequate doses and rampant overuse contribute to the failure of antibiotics in treating chronic wounds; all of which stem from inaccurate screening methods. Treatment plans based on a biofilm relevant assessment will allow clinicians to implement a biofilm-based
treatment plan and provide better clinical outcomes overall. The success of the WLM is its ability to allow biofilm formation, sustain relevant polymicrobial communities and consequently mimic a relevant antibiotic tolerance scenario. We urge other microbiologist and medical practitioners to consider these key factors when developing and choosing models for future research or diagnostics of infectious diseases.

Acknowledgements

We would like to thank the members of the Section of Microbiology for critical discussion of this manuscript.

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was supported by The Danish Council for Independent Research grant 11106571.

ORCID

Urvish Trivedi http://orcid.org/0000-0003-1541-6212
SørenJ. Sørensen http://orcid.org/0000-0001-6227-9906

References


European Centre for Disease and Control. (2014). Surveillance report – antimicrobial resistance surveillance in Europe


Manuscript 2
The sociobiology of coagulases, two distinct public goods of *Staphylococcus aureus* in a clinically relevant *in vitro* infection model

Urvish Trivedi¹, Jonas S. Madsen¹, Jake Everett², Cody Fell², Jakob Haaber³, Alexander Horwill⁴, Mette Burmølle¹, Kendra P. Rumbaugh⁵, Søren J. Sørensen¹

¹ Section of Microbiology, Department of Biology, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

² Department of Surgery, Texas Tech University Health Sciences Center, Lubbock Texas, United States of America

³ Department of Veterinary and Animal Sciences, Faculty of Health & Medical Sciences, University of Copenhagen, Copenhagen, Denmark

⁴ Department of Immunology and Microbiology, Anschutz Medical Campus, University of Colorado, Aurora, Colorado, United States of America

Abstract

Coagulation is an ancient innate defense mechanism intended to limit blood loss and trap invading pathogens during infection. However, as with other host defense mechanisms, *Staphylococcus aureus* has the ability to hijack the coagulation cascade and generate clots as part of its own survival strategies. *S. aureus* secretes two coagulases that are able to mediate the clotting of blood and plasma, staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp). Although this trait has been a diagnostic marker for identifying *S. aureus* for over a century, the social evolution theories and population dynamics regarding coagulases have yet to be explored. We report here that coagulases, like many other extracellular factors that promote bacterial growth and virulence, are public goods that interact with the surrounding host physiological components and confer protection within a local group or population. By utilizing isogenic variants, we were able to infer that the secretion of coagulases is a cooperative trait, which is subject to being exploited by invading mutants that no longer share the interests in producing the public goods themselves. These findings emphasize the critical role of coagulases in a social evolution context and suggest that therapeutic strategies targeting these public goods might be a viable approach for reducing the severity of *S. aureus* infections.
Introduction

The use of intravenous catheters has become widely accepted in clinical practice for long-term venous access in patients requiring continuous perfusion [1-5]. Intended for the administration of fluids, blood transfusions, medications, nutritional support, chemotherapy and hemodynamic monitoring [1-3, 5], these devices have become a major source of healthcare-associated infections. The impact of catheter-related bloodstream infections is substantial, both in terms of morbidity and financial resources expended [6-11]. Since intravenous catheters are in direct contact with the bloodstream, their surfaces become coated with plasma, platelets and extracellular matrix (ECM) proteins such as albumin, fibrinogen, fibronectin, and laminin [12], making the host vasculature highly susceptible to microbial colonization and biofilm formation [13]. Typically, biofilms are regarded as communities embedded in a bacterial-produced matrix where the extracellular polymeric substance (EPS) is composed of self-secreted polysaccharides, proteins, and DNA. However, this is rarely the case during infections, where pathogens are able to sense environmental cues and utilize the surrounding ECM components to form a host-derived matrix (HDM).

*Staphylococcus aureus* is one such versatile opportunistic pathogen that is frequently associated with endovascular infections. Its ability to interact with ECM components via secreted factors is a defining feature of *S. aureus* infections, where a vast array of its virulence genes coding for adhesins/invasins, toxins and modulins contribute to its colonization, dissemination, and persistence in host tissue. However, certain staphyloccocal factors are secreted out into the surrounding environment, where they specifically interact with the aforementioned ECM components found in blood. In terms of sociobiology, these secreted factors can be regarded as ‘public goods’ that benefit all individuals present within a local group or population. Biofilm development and maintenance *in vivo* is often dependent on and can be influenced by public goods [14]. Granted the expression of these public goods can prove to be costly to the producer, they provide a communal benefit and can, therefore, be potentially selected for by the overall population. However, this type of behavior poses an evolutionary conundrum because it is vulnerable to being exploited by ‘free-riders’ or cheats that do not cooperate in producing the public goods but can still benefit at the expense of producers. This dilemma is well known in the fields of economics and human morality, where it is termed the *tragedy of the commons* [15]. The tragedy is that as a group, individuals stand to benefit from cooperation, but cooperation is not stable because each individual can gain by selfishly pursuing their own short-term interests. Despite the merits of investigating these mechanistic and evolutionary theories in a host-pathogen context, little is known about the role of public good virulence factors during infections and their influence on population dynamics. Therefore, with an interdisciplinary approach, we use a highly relevant *in vitro* clinical model to explore the theoretical underpinnings of the social dynamics occurring during staphyloccocal bloodstream infections. We focus on the two known coagulases of *S. aureus*, staphylocoagulase (Coa) and von Willebrand factor-binding protein (vWbp).

Coa and vWbp are two hemostasis factors that allow *S. aureus* to usurp the physiological blood coagulation cascade. Both coagulases use molecular sexuality mechanisms to trigger a conformational change and induce a functionally active catalytic site in the host coagulation zymogen, prothrombin (ProT) [16]. The enzymatically active staphylothrombin complexes (ProT•Coa and ProT•vWbp) then facilitate clotting of plasma by cleaving fibrinogen from its substrate form to insoluble polymerizing fibrin fibrils. This dense fibrous
clot is further strengthened by the crosslinking activity of the transglutaminase factor XIIIa, a fibrin-stabilizing enzyme that is nonproteolytically activated by the ProT•vWbp•fXIII complex [17]. However, given that Coa and vWbp are vital during infection, an enduring question is: Why are changes in gene expression of coagulases observed? Although no single mechanism is responsible, phenotypes of low expression levels, loss-of-function mutations, and/or complete deficiency of coagulases are observed among clinical isolates of *S. aureus*. For example, Young and colleagues found cardiac device infections to be strongly associated with single nucleotide polymorphisms in the *coa* gene [18]. Therefore, we propose that secretion of coagulases is a defining feature of *S. aureus* fitness during infection, but it is also a costly and potentially exploitable trait. Our model organisms include: a community-acquired methicillin-resistant *S. aureus*, USA300 LAC, that produces Coa and vWbp; an isogenic Δcoa mutant that does not produce Coa; and an isogenic ΔcoaΔvwbp double-mutant that does not produce Coa and vWbp. The isogenic variants, Δcoa and ΔcoaΔvwbp, represent cheats that do not produce the public goods of interest, whereas LAC represents producers.

**Results**

Coagulases mediate clotting of wound-like media

Previous work established a simple yet elegant *in vitro* model for studying pathogens found in clinical infections [19, 20]. Termed ‘wound-like’ media (WLM), this model is formulated to represent physiological components encountered within blood and host vasculature. Previously, it has been used to study polymicrobial populations found in human wounds and the efficacy of various antimicrobials as potential treatments [19, 21-23]. Here, we utilize the WLM to mimic biofilms and septic thrombi associated with catheter-related bloodstream infections. In order to supply a more infection-like environment, we constitute the WLM with a chopped-meat-based medium, heparin-treated plasma, and red blood cells. Heparin is a glycosaminoglycan that effectively inhibits the clotting of blood, and is frequently used in clinical practice as a prophylactic measure against thrombosis. The anticoagulant properties are mediated by heparin’s interaction with the enzyme inhibitor antithrombin that inactivates thrombin, factor Xa, and other proteases, thereby preventing the endogenous conversion of fibrinogen to fibrin clots [24]. Despite therapeutic doses of heparin being commonly administered alongside venous catheter installations, septic thrombi continue to be a major concern for patients where bacteria circumvent the proteolytic process of blood coagulation. Thus, the first aim of our study was to utilize the heparinized WLM to identify potential public goods of *S. aureus* that facilitate clotting and form thrombi similar to those observed *in vivo* (Fig. S1).

We initially screened the clotting ability of various *S. aureus* proteins using a knock-out library. Inoculation of fresh heparin-treated WLM with *S. aureus* LAC triggered clotting, whereas the ΔsaePQRS mutant was unable to generate clots within 24 hours (Fig. S2A). The Sae regulatory system (*S. aureus* exoprotein expression) controls the expression of several genes encoding proteins known to interact with host ECM components [25]. Therefore, we screened various *S. aureus* factors that could potentially mediate clotting of the WLM: fibronectin binding proteins (FnbpA and FnbpB) that bind to fibrinogen/fibrin [26, 27]; clumping factor (ClfA) that binds to fibrinogen/fibrin [28]; extracellular matrix and plasma binding protein (Empbp) that interacts with fibrinogen/fibrin and vitronectin [29]; extracellular adherence protein (Eap) that binds to fibrinogen, fibronectin, vitronectin, thrombospondin, and collagen [30, 31], and the prothrombin-activating proteins Coa and vWbp.
With the exception of ClfA, most, if not all of these genes are transcriptionally activated by the saeRS two-component system [25, 34, 35], of which Coa was found to mediate the clotting of the WLM (Fig. S2B). Disruption in any of the other genes did not affect the ability of *S. aureus* to clot the WLM (Fig. S2A and B).

These phenotypic data led us to construct our final working strains: USA 300 LAC, Δcoa, and ΔcoaΔvwp double mutant. Inoculation of fresh heparin-treated WLM with LAC triggered clotting, whereas the isogenic Δcoa and ΔcoaΔvwp mutants were unable to generate clots within 24 hours. We also constructed a Δvwp mutant that was able to clot the WLM within 24 hours, and therefore excluded from the study. This observation was most likely because of Coa compensating for the lack of vWbp. However, vWbp is known to form a functionally active complex with ProT, fibrinogen, and factorXIII [17]; and is suggested to compensate for the lack of Coa during endocarditis [36, 37]; hence, the inclusion of the ΔcoaΔvwp double mutant within our study. Therefore, in our study, coagulases, Coa and vWbp, are the potential public goods that contribute to the ability of *S. aureus* to generate robust clots (Fig. 1).

### Cheats coexist in close proximity to the producers within clots

*In vivo*, pathogenic bacteria often grow as non-surface associated aggregates that are found interspersed throughout the infected host tissue [38-49]. Typically, studies focus on visualizing bacterial glyocalyx; however, this is equivocal, considering *in vivo* biofilms are a combination of both the bacteria-derived EPS and HDM [50]. This is the case with septic thrombi, where secreted coagulases allow *S. aureus* to utilize the ECM substrates to generate clots incorporating the HDM as part of the biofilm. To verify this phenomenon, we used a lectin dye, concanavalin A (ConA) that does not discriminate between the EPS and HDM to provide an accurate spatial context of our *in vitro* model. Due to its ability to selectively bind α-mannopyranosyl and α-glucopyranosyl residues, we have previously used ConA to stain matrix components in sections from infected wounds [51].

![Figure 1. Clotting of WLM is facilitated by *S. aureus* coagulases.](image)

Heparin-treated WLM was inoculated with LAC, or its isogenic variants Δcoa, Δvwp, or ΔcoaΔvwp and incubated for 24 hours at 37°C. Cultures were poured into a petri dish to assess coagulation. Data are representative of three different determinations.

Thin-sections of coagulated WLM that had been cocultured for 24 hours with LAC and Δcoa, or LAC and ΔcoaΔvwp were stained and visualized using confocal laser scanning microscopy. Micrographs revealed clumps of cheats and producers coaggregating indiscriminately, enmeshed in a web of fibrous matrix (Fig. 2). The staphylococcal aggregates were interspersed throughout the fibrous HDM, all the while enclosed and segregated by fibrin strands. The HDM served as a scaffold to which the bacteria could adhere and reside within. It should be reiterated that monocultures of cheats are unable to coagulate the WLM; therefore the observed fibrous
architecture is orchestrated only in the presence of producers, where coagulases induce fibrinogen cleavage to polymerize protective layers of fibrin around the core of staphylococcal aggregates. Taken together, these micrographs indicate that cheats are able to integrate themselves into the fibrin clots generated at the expense of the producers. It should be noted that here we regard coagulases as public goods that act on the surrounding host ECM in generating clots, and not directly on the surrounding bacterial cells. Therefore, we decided to measure a classical parameter that is both dependent on community structure and biofilm formation, antimicrobial tolerance.
Access to coagulases confers enhanced antimicrobial tolerance

Prompt administration of intravenous antibiotics is one of the principle treatments for patients suspected of developing septic thrombi [5, 52]. Despite conservative measures, evidence of a septic thrombus often requires removal of infected cannulas and/or surgical intervention because antibiotics alone often prove to be insufficient in resolving the infection. Biofilm-related antimicrobial tolerance has been observed in vivo and in vitro; therefore, having visualized the marked phenotypic differences in the clotting ability of our staphylococcal strains, we sought to determine if the expression of or having access to public goods affected their antimicrobial susceptibilities. Our experiments examined the consequences of variation for a single trait, whether or not they produce coagulases. In the monoculture groups, WLM was inoculated with only the LAC, Δcoa, or ΔcoaΔvwbp. Whereas in the coculture groups, WLM was inoculated with a 1:1 mixture of both the LAC and Δcoa, or LAC and ΔcoaΔvwbp. Monocultures and cocultures were grown overnight in WLM, and antimicrobial tolerance was determined as described in Materials and Methods. We assessed the tolerance of S. aureus strains to an aminoglycoside (gentamicin) and an antimicrobial (ethanol). As shown in Figure 3, monocultures of producers (LAC) displayed higher tolerance to both gentamicin and ethanol in comparison to the monocultures of cheats (Δcoa and ΔcoaΔvwbp). Notably, the cheats displayed an increase in tolerance to both gentamicin and ethanol when present in cocultures with producers in comparison to their monocultures.

Given the increase in biomass associated with biofilms or lack thereof, it is reasonable to claim that the observed tolerance profiles are owed to the thrombus generated in the presence of coagulases. Monocultures of cheats are unable to clot the WLM and thereby do not generate the HDM, leaving them susceptible to antimicrobials. However, when cocultured with producers, cheats are incorporated into the septic thrombi, whereby secreted coagulases precipitate fibrinogen cleavage and fibrin clots surrounding the staphylococcal communities (Fig. 2). Therefore, it is important to state that here the benefits of public goods accrue not only to the producers within the community but also to the cheats residing therein.
This distinction is important in terms of sociobiology because it leads to the fundamental question of what favors the cooperative production of coagulases.

**Stressors can select for cooperators and defer a tragedy of the commons**

Septic thrombosis presents an acute clinical complication that can result in lethal outcomes if not treated promptly [53]. Therefore, due to the associated mortality risks, it is impossible to conduct longitudinal studies of underlying microbial interactions within patients. Thrombosed catheters are removed immediately, and routine administration of intravenous antibiotics and/or anticoagulants are initiated to prevent recurring clots. Despite these efforts, *S. aureus* is able to reconstitute the thrombus and persist within the host vasculature. Here we used the WLM to investigate how stressors influence the relative fitness of producers v. cheats within the septic thrombi. In this assay, we follow the dynamics over several days and generations as opposed to the assay used in the previous results.

The population dynamics experiment contained two treatment groups: phosphate buffered solution (PBS) and gentamicin. The variation in our experiment is with respect to coagulation, which is the trait, whose evolution we are interested in. We were able to focus on coagulases because the producers and cheats are initially identical at other parts of the genome, and so there is no genetic variation for other traits to influence selection. We initiate the experiment by inoculating the heparinized WLM with a 1:1 mixture of LAC and Δcoa or LAC and ΔcoaΔvwbp. After 24 hours of growth, the WLM cultures were subjected to parallel but different treatments (PBS or gentamicin). Then, all subsequent rounds were initiated with the treated population from the preceding round. In order to calculate the relative frequency of producers v. cheats, bacteria were enumerated after each round of treatment. As we propagated the bacterial population through the different selection rounds, we found that the cheats were favored under conditions of PBS treatment (Fig. 4). This trend held irrespective of whether we analyzed the cocultures harboring Δcoa or ΔcoaΔvwbp. Intuitively this makes sense, as cheats are not taxed with the costs of producing coagulases, they can divert resources towards other metabolic activities and can thereby increase in frequency.

![Figure 4. Consequential penalty resulting from over-exploitation defers a tragedy of the commons.](image)

Cocultures of USA300 LAC and Δcoa, or LAC and ΔcoaΔvwbp were grown in heparin-treated WLM for 24 hours at 37°C. The population from these cultures was then subjected to PBS or gentamicin treatment and propagated through multiple days of culturing. Population ratios were assessed by bacteria enumeration of colony forming on selective agar plates. Data represent three independent samples.
Put simply, coagulases are not advantageous in conditions without stressors, where producers and cheats can interact, allowing cheats to exploit the common resources and gain a competitive advantage.

In contrast, the producers were favored under conditions of gentamicin treatment (Fig. 4). When producers were the majority of the population, cooperation was maintained, thereby making the Coa and vWbp dependent fibrous matrix sustainable. This is important because the overall staphylococcal community stands to benefit when the structural integrity of the septic thrombus remains uncompromised. Whereas a higher frequency of cheats delays clotting of the WLM, resulting in a more fluid biofilm, leaving the community more susceptible to gentamicin. In addition, since cheats do not clot the WLM, one can expect the fluid layers to harbor more cheats than producers. Therefore, cheats are kept in check by the stressor and a tragedy of the commons is deferred because exploitation of producers results in immediate consequential penalties. Undoubtedly, access to public goods confers benefits within the WLM; however, as our in vitro model lacks the immunological factors encountered within blood, we wished to see if the benefits of secreted public goods could be recapitulated in fresh human blood.

**Staphylococcal survival in human blood**

The host complement system provides an immediate response via a highly specific and regulated sequence of proteins binding to invading pathogens. The abundant complement proteins in blood serum form membrane attack complexes (MACs) that open rupturing pores in microbial cell membranes or serve as chemoattractants that are markers for phagocyte migration and enhanced opsonization of targeted pathogens [54-56]. In addition to the antimicrobial peptides and MACs that mediate bacterial killing, upon pathogen recognition, polymorphonuclear neutrophils (PMNs) and monocytes are able to quickly engulf *S. aureus* and commence digestion via acidic vacuoles, reactive nitrogen compounds, and reactive oxygen species. For this reason, we analyzed the contribution of coagulases towards staphylococcal survival in fresh heparin-treated human blood. As before, monoculture groups were inoculated with only the LAC, Δcoa, or ΔcoaΔvwbp. Whereas the coculture groups were inoculated with a 1:1 mixture of both the LAC and Δcoa, or LAC and ΔcoaΔvwbp. Monoculture of LAC displayed a very slight reduction in CFU in human blood (Fig. 5). In contrast, the monocultures of the mutants, Δcoa and ΔcoaΔvwbp, suffered a large reduction in CFU, exhibiting a defect in survival in human blood (Fig. 5). Interestingly, coculturing the cheats with producers significantly improved the survival of both Δcoa and ΔcoaΔvwbp in human blood (Fig. 5).

**Figure 5. Access to coagulases enhances staphylococcal survival in blood.** USA300 LAC as well as its variants Δcoa, or ΔcoaΔvwbp were incubated in heparin-treated human blood for 45 (dark grey) or 90 (light grey) minutes and bacterial survival assessed by bacteria enumeration of colony forming units on selective agar plates. Data generated from three independent trials (* p<0.05 by analysis of ANOVA test for the 90 min data).

This trend held for both time points analyzed in our experiment. These data indicate that coagulases function as public goods and
enhance staphylococcal survival in human blood, where Coa and vWbp generate fibrin shields that contribute towards masking complement factors, thereby interfering with the formation of MACs and interfering with the phagocytic uptake of bacterial cells.

Discussion

A key aspect of this work was the utilization of relevant infectious models in identifying coagulases as public goods that can be exploited. Many studies that focus on the sociobiology of biofilms are carried out in liquid media that bear little- or no clinical relevance whatsoever. Therefore, the contributions of public goods towards the pathogenesis of staphylococcal infections in the presence of ECM components, but not in their absence, are overlooked. Generally, septic thrombi are not considered biofilms, where coagulation and clot formation are regarded as part of the host’s innate defense mechanism. However, these infections often involve aggregates of bacterial cells in a milieu of HDM, with the bacteria harnessing the surrounding ECM proteins to protect themselves. Therefore, with the growing number of biofilm-related infections and emerging knowledge, the term ‘biofilm’ is constantly being redefined to incorporate new findings. S. aureus is one such pathogen that is able to co-opt the physiological coagulation cascade and utilize host proteins as part of its immune evasion and survival strategy during infections. In that sense, we propose that septic thrombi are a form of HDM-biofilms that culminate in the presence of select staphylococcal virulence factors. During these events, interactions with the abundant host plasma glycoproteins fibrinogen, fibrin, and fibronectin are particularly important. Although FnbpA, FnbpB, ClfA, Emnbp, and Eap are known to interact with these ECM components, their binding to fibrinogen does not precipitate fibrinogen cleavage and/or fibrin clot formation. The ability to catalytically convert fibrinogen to fibrin and generate clots is attributed to our primary public goods of interest, Coa and vWbp. As the activities of these two proteins partly overlap, they may seem functionally redundant at first glance. However, this is not the case. Both coagulases share structural homology within the N-terminal half of the protein that binds prothrombin [32], whereas the C-terminal half of vWbp differs from that of Coa, where vWbp includes a unique binding site for the blood glycoprotein, von Willebrand factor [33]. In addition, vWbp displays lower binding affinity to ProT than Coa, where the subsequent ProT•vWbp complex displays different catalytic activity than ProT•Coa and generates fibrin monomers at a reduced rate [16]. Perhaps the enzyme kinetics explains the disparity in the coagulation of our WLM, where the Δvwbp mutant was able to generate clots within 24 hours, but Δcoa did not. Regardless, the fact that both staphylothrombin complexes interact with host fibrinogen is an important attribute.

Fibrinogen is an abundant plasma glycoprotein that is present at concentrations of 2-4 mg/mL (6-12 µM) in blood and is a central player in the clotting cascade [57]. The role of fibrinogen and fibrin during infection are complex. Intended to promote containment and clearance of infectious foci by the immune system, S. aureus takes advantage of the host fibrinogen present at the site of infection for its own survival. Undoubtedly, fibrinogen was shown to be an important constituent that shields S. aureus from host defenses [58, 59]. However, the presence of this physiological constituent was a commonality in our human blood samples and WLM cultures, where the observed staphylococcal survival and antimicrobial tolerance profiles in our experiments cannot be attributed to fibrinogen alone. Therefore, with the initial ECM components being identical, the marked difference between the cultures after inoculation remains the clotting of WLM and
the human blood mediated by Coa and vWbp. Coagulases secreted by S. aureus catalyze fibrinogen cleavage and enclose the pathogen in protective layers of fibrin cables (Fig. 2). Therefore, the presence of fibrinogen-rich clumps is most likely a prerequisite for the subsequent coagulation and fibrin polymerization.

One of the major hallmarks associated with biofilm infections is their increased tolerance to antimicrobials. In the case of staphylococcal infections, this common theme is attributed to the presence of fibrin deposits within the biofilm [60, 61]. Using the WLM model, we observed this phenomenon in our antibiotic tolerance assay (Fig. 3). Monocultures of producers residing in coagulated WLM were significantly more tolerant to gentamicin and ethanol in comparison to the cheats that were unable to generate clots (Fig. 3). Notably, after being cocultured with producers in coagulated WLM, this protective effect was also conferred onto the cheats. In addition to the producers being fortified, aggregates of cheats were also incorporated within the polymerized fibrin meshwork (Fig. 2). The penetrating ability of gentamicin is dependent on the fundamental properties of electric charge, where the basophilic HDM poses a challenge for the positively charged antibiotic. Tseng et al. demonstrated that the positively charged antibiotic tobramycin became sequestered in the negatively charged biofilm periphery, while the neutral antibiotic ciprofloxacin readily penetrated them [62]. Therefore, the poor efficacy of gentamicin in our tolerance assay most likely owes, in part, to its inability to access the fibrin-encased staphylococcal aggregates. We further demonstrate the benefit conferred upon access to coagulases in human blood as well, where S. aureus must overcome major hurdles posed by the innate immune response such as antimicrobial peptides, complement and phagocytic killing [63, 64]. Entrapment and clearance of infection by the host immune system depends on the binding of complement proteins, pathogen recognition, and the ability of phagocytes to access opsonins. Monocultures of producers displayed much higher survival rates in human blood compared to monocultures of cheats that had the lowest survival rates (Fig. 5). However, the survival of cheats improved dramatically upon being cocultured with producers that were able to generate clots (Fig. 5). Our observations align with other studies that have demonstrated that fibrin-encased bacteria do not activate immune cells and can thus escape phagocytosis [60, 65]. Earlier work suggests that Coa is probably responsible for the formation of a fibrin shield directly around bacterial cells, while vWbp induces fibrin formation towards the periphery of the staphylococcal community [66, 67]. We surmise that the fibrinogen and fibrin coat formed in the immediate vicinity, prevent attachment of complement proteins and antimicrobial peptides; whereas those formed distal to the staphylococcal aggregates inhibit opsonin recognition by immune cells and act as a physical obstacle for incoming phagocytes. Most antimicrobial peptides circulating in blood have a positive charge so that they can bind the negatively charged cell-surfaces of bacteria. However, akin to the same way gentamicin becomes sequestered, the positively charged antimicrobials most likely become sequestered in the negatively charged biofilm as well. Together, these barriers generated by Coa and vWbp interfere with the complement activation, binding of antimicrobials, formation of MACs, and opsonophagocytosis of the fibrin-encased staphylococci.

Of course, the benefits of the ensuing coagulation product rely on the fact that the cheats situate themselves in close proximity to the producers, where the concentration of public goods is likely to be high. In S. aureus, clumping is mediated via the regulator systems, MgrA and ArlRS [68]. We deem this to be an important trait as it allows cheats to clump with producers, a phenomenon evidenced in our
micrographs, where coaggregates of both cheats and producers were incorporated indiscriminately within the fibrin meshwork (Fig. 2). Coaggregation allows cheats to place themselves in the immediate vicinity of the public goods and subsequently maximize their benefit and exploitation of the secreted coagulases. Cooperative production of public goods is associated with fitness costs that divert resources away from primary metabolism. Hence, co-aggregation can lead to competition for resources between producers and cheats. This competition over resources is observed to a certain degree in our antibiotic tolerance assay as well as staphylococcal survival in human blood, where the fitness of the producers decreases slightly when cocultured with cheats in comparison to being present in monocultures (Fig. 3 and 5). Monoinfection of LAC harbors only producers that cooperatively secrete coagulases; thus, the costs of producing public goods are shared. Whereas, in coinfections, which harbor both producers and cheats, the Δcoa or ΔcoaΔvwbp benefit from the coagulases secreted by the LAC without incurring the costs of producing the public goods themselves. This phenomenon is evident in our relative fitness assay, where cheats do not incur the costs of secreting coagulases and therefore increase in frequency, as they out-compete the cooperative producers (Fig. 4A). However, the repeated exposure to gentamicin defers a tragedy of the commons, where the producers retain the majority (Fig. 4). We hypothesize that population dynamics are regulated by the spatial organization and limited dispersal, rather than active communication and coordination among constituent members. In other words, as cheats increase in frequency, the overall community transitions from being in a well-established thrombus to a partially coagulated culture that is more fluid in nature, where the weaker parts of the biofilm harbor more cheats than producers. Therefore, staphylococci within these weaker parts are eradicated/out-selected and thereby not passed onto the next round of culturing, allowing the producers to retain the majority and coagulation to be selected for. These results suggest that cheating is advantageous in situations where costly public goods are readily accessible and cooperation isn’t favored.

**Materials and Methods**

**Bacterial strains and growth conditions**

Non-fluorescent strains were used in experiments unless otherwise stated. Strains were grown in Tryptic soy broth (TSB) with 100 µg/mL rifampicin or streptomycin prior to inoculation of WLM or human blood. Enumeration of colony forming units was done on selective Tryptic soy agar (TSA) plates infused with 20 µg/mL rifampicin or streptomycin.

**Construction of strains**

Regions upstream and downstream of coa were amplified using primers HC375/HC376 and HC377/HC378, and the pJB38 backbone was amplified using primers HC367/HC368. The resulting fragments were fused using the Gibson assembly master mix (New England Biolabs), generating plasmid pH83. A plasmid for deleting vwbp, pH85, was generated by the same method, using primers HC387/HC370 and HC371/HC372. Deletion plasmid were electroporated into *S. aureus* RN4220, selecting on TSA plates containing Cam (10 µg/ml) at 30 °C. The plasmids were then transduced into *S. aureus* strain LAC. Individual colonies were streaked on TSA Cam plates incubated at 42 °C to select for integration into the chromosome. Single colonies were grown in TSB at 30 °C and diluted 1:500 in fresh media for four successive days before diluting to 10^{-6} and plating on TSA containing 0.3 µg/mL anhydrotetracycline to select for loss of the plasmid. Colonies were screened for sensitivity to Cam, and Cam^S colonies were screened by PCR for deletion of coa or vwbp. Refer to Fig. S3 for oligonucleotide sequences.
**Fluorescent tagging of strains**

USA300 LAC wild-type (AH1263) was tagged with green fluorescence by transferring plasmid pCM11 carrying sGFP from strain AH1331 yielding strain JH992. AH1263 was also tagged with mCherry by transferring plasmid pAH9 from strain AH478, yielding strain JH991. Likewise, USA300 LAC coa mutant (AH4035) was tagged by transferring pCM11 and pAH9 yielding strains JH994 and JH993, respectively. The plasmids were transferred between strains using standard phage transduction protocols outlined by Olson, M.E. [69].

**Screening phenotypes using the wound-like media**

The wound-like medium (WLM) consists of 45% Bolton broth, 50% bovine plasma, and 5% laked horse red blood cells. Glass 16 x 100 mm test tubes with caps were autoclaved, and 4 mL biofilm formulation media was aseptically dispensed into each tube. The overnight cultures were centrifuged for 10 min at 7000 rpm and re-suspended in the same volume of 1x phosphate-buffered solution (PBS), repeated twice to rinse all antibiotics. Then the optical densities of all overnight cultures were normalized for all of the strains. The glass tubes were then inoculated with 10 µL of 10⁴ to 10⁵ CFU/mL of \( S. aureus \) mono- or cocultures. The tubes are then incubated at 37°C for 24 h on an orbital rotator at 50 rpm.

**Staining and imaging**

WLM was prepared in the same manner as described above, with the exception of 460 µL volume being placed in a 5 cm x 0.5 cm glass tube, and inoculated with 7.5 µL of 10⁴ to 10⁵ CFU/ml of \( S. aureus \). Imaging was performed on frozen sample sections. Briefly, coagulated WLM was removed from tubes, placed in a Tissue-Tek vinyl specimen Cryomold (Sakura Finetek, Torrance, CA, USA) containing a cryomatrix of OCT (optimum-cutting-temperature) compound (Thermo Fisher Scientific, Kalamazoo, MI, USA), and then immediately placed in a freezer at –80°C to allow the OCT compound to solidify. Frozen sections were securely anchored using deep-waffled, large face block holders (Electron Microscopy Sciences, Hatfield, PA, USA). Frozen OCT-embedded samples were sectioned using an OTF5000 cryostat (Bright Instrument Co., Ltd., Huntingdon, Cambridgeshire, England) to a thickness of 6-8 µm and were then directly transferred to Superfrost Plus microscope slides (Thermo Fisher Scientific) and stored at –20°C until ready for visualization. Frozen sample sections were prepared for staining by air drying at room temperature for 5 min, washed three times in 1x PBS (2 min each time), fixed in 4% paraformaldehyde at room temperature for 15 min, washed three times in 1x PBS (2 min each time), allowed to air dry at room temperature for 5 min before addition of the stain. Matrix components were visualized by staining sections with 100 µg/mL Texas Red-conjugated concanavalin A (ConA) (Invitrogen, Carlsbad, CA, USA) in the dark for 5 min at room temperature, washing three times in 1x PBS (5 min each time), and then mounting with Prolong Gold Antifade reagent (Molecular Probes, Eugene, OR, USA) supped with 4', 6'-diamidino-2-phenylindole (DAPI) to stain DNA. Images were acquired with a Nikon T1-E microscope with A1 confocal and STORM super-resolution modules, and images were captured with an EMCCD camera (iXon Ultra 897, Andor, DU-897E-CS0-#BV) and analyzed with the NIS Elements program (version 4.51.01, Nikon, Japan).

**Antimicrobial tolerance assay**

Sections of coagulated WLM or 460 µL of uncoagulated planktonic WLM culture were suspended in 1 mL of 300 µg/mL gentamicin, 35 % ethanol, or 1x PBS for 5 h. Samples were then centrifuged and re-suspended in 1 mL of Dey-Engley broth and allowed to sit for 10 min. After which they were centrifuged and re-suspended in 1 mL of 1x PBS, homogenized, vortexed, serially diluted, and plated on Tryptic soy agar (TSA) with 20 µg/mL rifampicin or...
streptomycin for enumeration of colony forming units. CFU was quantified per milliliter or per gram, respective to the samples. Even though Dey-Engley broth is frequently used to neutralize antiseptics in order to avoid false-negative results due to drug carryover; to our knowledge, its neutralizing properties do not extend to antibiotics. Therefore, in our assay, it was used as a rinsing agent.

**Relative fitness assay**

WLM media was prepared as described above, and 460 µL of uncoagulated planktonic culture or sections of coagulated WLM were suspended in 1 mL of 1x PBS for 5 h, homogenized, vortexed, serially diluted and plated on selective TSA agar for enumeration of colony forming units. CFU was quantified per milliliter or per gram, respective to the samples. The 10-2 dilution (10^4 – 10^5 CFU/mL) of the WLM homogenate was used to inoculate fresh WLM; this procedure was repeated every 24 h, while propagating the bacteria through multiple days of culturing.

A similar setup was used for the gentamicin treated rounds, with the exception that the 460 µL of uncoagulated planktonic culture, or sections of coagulated WLM were suspended in 300 µg/mL gentamicin for 5 h. Samples were then centrifuged and re-suspended in 1 mL of Dey-Engley broth and allowed to sit for 10 min. After which they were centrifuged and re-suspended in 1 mL of 1x PBS, homogenized, vortexed, serially diluted, and plated on selective TSA agar for enumeration of colony forming units. CFU was quantified per milliliter or per gram, respective to the samples. The homogenate was used to inoculate fresh WLM for the following day; this procedure was repeated at every 24 h, while the propagating the bacteria through multiple days of culturing.

**Blood survival assay**

Overnight cultures were centrifuged for 10 min at 7000 rpm and re-suspended in the same volume of 1x phosphate-buffered solution (PBS), repeated twice to rinse all antibiotics. The optical densities of all cultures were normalized for all of the strains to generate a suspension of 1x 10^7 CFU/mL. Fresh whole blood was collected from consenting human volunteer by venous puncture using a 0.6 x 19 mm x 305 mm and 23G x 3/4” x 12” push button blood collection set (Becton Dickinson); 4 mL of blood was collected into 13 x 75 mm vacutainers with 75 USP units of freeze-dried sodium heparin (Becton Dickinson). 450 µL of blood was aliquoted into a 1 mL Eppendorf tube and inoculated with 50 µL of bacteria sample (1x 10^5 CFU/mL) from the monoculture or coculture suspensions. Samples were incubated at 37°C with slow rotation. 100 µL aliquots were removed at times 0, 45, and 90 minutes, mixed 1:1 with fresh 2% saponin/PBS and incubated on ice for 30 minutes. Five 1:10 serial dilutions were prepared and 100 µL aliquots spread on selective TSA agar for enumeration of colony forming units.

**Ethics statement**

The protocol for venous blood collection was approved by the Texas Tech University Health Sciences Center (TTUHSC) Institutional Review Board. Consent was obtained from healthy volunteers as mandated by the Clinical Research Institute (CRI) at TTUHSC in compliance with ethical practices. No admitted patients or children were involved in this study.

**Acknowledgements**

We thank Lauren Choate, Anette Løth for technical assistance and Sarah Julsrud for text revisions. Images were generated in the Image Analysis Core Facility supported in part by TTUHSC. This work was supported by the Danish Council for Independent Research grant 11106571.
References


18. Young BC, Kevin; Wu, Chieh-Hsi; Seifert, Harald; Rieg, Stegbert; Eduardo Lopez-Cortes, Luis; Gurgu, Mercedes; Lepe, Jose Antonio; Kim, Hong Bin; Park, Wan Baeom; Tilley, Robert; Scarborough, Matthew; Edgeworth, Jonathan; Llewelyn, Martin; Wilson, Daniel; Kaasch, Achim Polymorphisms in coagulase of Staphylococcus aureus are associated with infection of cardiovascular devices. 27th ECCMID; Vienna, Austria2017.


Supplementary information

Supplementary Figure 1. Visual comparison of the fully coagulated wound-like media with a thrombus taken from a patient. This image compares the fully coagulated wound-like media (left) to a manually aspirated thrombus (right) showing the similarity in texture and consistency between the in vitro model and the in vivo sample.

Supplementary 2A.

Supplementary 2B.
Supplementary 2. Screening potential public goods of Staphylococcus aureus that mediate clotting of WLM. Heparin-treated WLM was inoculated with USA 300 LAC, knock-out mutants, or transposon mutants were incubated for 24 hours at 37°C. Cultures were poured into a petri dish to assess coagulation. Data are representative of three different determinations.
**Oligonucleotides:**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC367</td>
<td>CGG GTA CCG AGC TCG AAT TCT TG</td>
</tr>
<tr>
<td>HC368</td>
<td>GGG ATC CTC TAG AGT CGA CCT GC</td>
</tr>
<tr>
<td>HC370</td>
<td>ATG ACC TTC ACT CTC GAG GGT ACC GCT AGC CAA CTG TAT TTT CTC CTT AAT TTT CCC</td>
</tr>
<tr>
<td>HC371</td>
<td>GAAAAATACAGTTGGCTAGCGGTACCCTCGAGAGTGAAGGTCATCAAAAA CGTTTTAAT</td>
</tr>
<tr>
<td>HC372</td>
<td>CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC TAA CTG TGA TTC TGG TAC GAC AAA GTT</td>
</tr>
<tr>
<td>HC375</td>
<td>CTTTCGTCITCAAGAATTCGAGCTCGGTACCCGTGGCAATTTGGCGTCAC AAA</td>
</tr>
<tr>
<td>HC376</td>
<td>GAG TTA CAA ACT TAC TCG AGG GTA CCG CTA GCT AAT GTC CCA TTT TTA CTC CCA GC</td>
</tr>
<tr>
<td>HC377</td>
<td>AAT GGG ACA TTA GCT AGC GGT ACC CTC GAG TAA GTT TGT AAC TCT ATC CAA AGA CAT A</td>
</tr>
<tr>
<td>HC378</td>
<td>CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGT TTA CTT AAT GAA ATG GGT AGA CG</td>
</tr>
<tr>
<td>HC387</td>
<td>CTTTCGTCITCAAGAATTCGAGCTCGGTACCCGGACCGGCTGGTGAATTTGA ACCAA</td>
</tr>
</tbody>
</table>

**Supplementary 3.** Oligonucleotide sequences for construction of the Δcoa and ΔcoaΔvwbp strains.
Manuscript 3
What's mine is yours: the essential and yet exploitable nature of coagulases during *Staphylococcus aureus* infection

Urvish Trivedi¹, Cody Fell², Jonas S. Madsen¹, Jake Everett¹, Mette Burmølle¹, Kendra P. Rumbaugh³, Søren J. Sørensen¹

¹Section of Microbiology, Department of Biology, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

²Department of Surgery, Texas Tech University Health Sciences Center, Lubbock Texas, United States of America

Abstract

*Staphylococcus aureus* is a prominent etiological agent of skin and soft tissue infections. Abscess formation and purulent discharge is a classical physiological feature of healing and tissue repair. However, *S. aureus* deploys two clotting factors, staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp), that are able to usurp this classical host response and promote abscess lesions that are distinctive for this pathogen. Using a clinically relevant *in vivo* murine model, we establish here that Coa and vWbp are shared public goods that are required for abscess formation and contribute to staphylococcal persistence. In support of this model, we examine the contribution of these public goods in a mixed *S. aureus* population and their consequences for virulence; staphylococci that are able to produce Coa and vWbp rescue mutants that no longer take part in synthesizing these public goods. Thus, by exploiting the already available Coa and vWbp, the mutants are able to thrive during infection at the expense of the producers. Although there is much interest in studying molecular mechanisms from a social evolution perspective, rarely do such studies employ accurate clinical models. This study shows the importance of social evolution theories regarding public goods and bridges them with the field of clinical sciences. These findings also provide possible explanations as to why coagulase-negative staphylococci may emerge and evolve during infections.
**Introduction**

*Staphylococcus aureus* is an infamous adversary of modern medicine. The pathogenesis of *S. aureus* is initiated upon its entry into tissues or the bloodstream via trauma, surgical wounds, or medical devices where it engages a large set of its virulence factors such as adhesin/invasins, pore-forming toxins, superantigens and immune evasion factors [1, 2]. These gene products contribute towards colonization, dissemination, tissue damage and transmission of *S. aureus*; however, a select few are specifically fine-tuned for establishing septic thrombi and abscesses [2-5]. The pathophysiology of staphylococcal infections encompasses a number of complex interactions, which are facilitated by a range of microbial social traits often involving the cooperative production of extracellular factors, such as enzymes and nutrient-scavenging molecules. These secreted products are considered ‘public goods’, whose production is energetically costly, but benefits all individuals present in a local group or population.

In terms of sociobiology, these cooperative traits pose evolutionary dilemmas because they can potentially give rise to ‘free-riders’ or cheats, who avoid the costs of producing the extracellular factors themselves, but are able to benefit from those produced by others [6-9]. Despite the appeal of exploring such concepts for future medical knowledge and strategies, there is a lack of accurate model testing in studying microbial interactions. The majority of empirical support for social interactions that occur in pathogenic microbial communities has come from studies employing nutrient-rich and minimal liquid media [10-13], making it unclear to what extent these interactions occur during infection, and if they are clinically relevant [14-16]. Here we examined bacterial infection in mice to test how access to public goods, either through cooperation or cheating, influences staphylococcal persistence within a murine abscess model. We focus on the two known coagulases of *S. aureus*, staphylocoagulase (Coa) and von Willebrand factor-binding protein (vWbp).

Coa and vWbp are hemostasis factors that activate the central coagulation zymogen, prothrombin (ProT), in a non-proteolytic manner. The resulting staphylothrombin complexes (ProT•Coa and ProT•vWbp) then convert soluble fibrinogen from its substrate form to the polymerizing insoluble fibrin strands and generate clots [17-20]. Unlike the host-initiated pathways, Coa and vWbp are not targeted by circulating thrombin inhibitors (e.g. heparin), which allows them to hijack the host clotting machinery by activating ProT independently and bypassing the physiologically regulated coagulation cascade [18, 20]. Despite this unique trait, atypical coagulase-negative *S. aureus* are isolated from infections. In addition, certain isolates display varying levels of defects regarding coagulases, ranging from low expression levels and loss-of-function mutations to complete deficiency. Given the stochastic nature of these defects, one can speculate that Coa and vWbp are essential but costly to produce and are therefore exploitable during the pathogenic life cycle of *S. aureus*. In relation to that, we explore the role of coagulases in a sociobiology context and their contributions as public goods for staphylococcal infections. Our model organisms include: a community-acquired methicillin-resistant *S. aureus*, USA300 LAC, that produces Coa and vWbp; an isogenic Δcoa mutant that does not produce Coa; and an isogenic ΔcoaΔvwbp double-mutant that does not produce Coa and vWbp. The isogenic variants, Δcoa and ΔcoaΔvwbp, represent cheats that do not produce the public goods of interest, whereas LAC represents producers.
Results

Coagulases enhance staphylococcal survival within human blood and WLM

Previous work established the wound-like media (WLM) as a clinically relevant in vitro model that mimics physiological concentrations of blood components [21-23]; we used it to study septic thrombi associated with *Staphylococcus aureus* infections. We measured the coagulation phenotypes of *S. aureus* LAC and the isogenic mutants in heparinized WLM. Clotting of blood and WLM is effectively inhibited by heparin, a glycosaminoglycan produced by basophils and mast cells, that results in a ternary complex between antithrombin, thrombin, and heparin [24]. The anticoagulation process is mediated by heparin’s interaction with the enzyme inhibitor, antithrombin, which inactivates thrombin, factor Xa and other proteases, thereby preventing the physiological conversion of fibrinogen to fibrin [24]. Inoculation of fresh heparin-treated WLM with LAC triggered clotting, whereas the isogenic Δcoa and ΔcoaΔvwbp mutants were unable to generate clots within 24 hours. The Δvwbp was able to clot the WLM within 24 hours, and therefore excluded from the study. Nevertheless, vwbp is known to form a complex with ProT, fibrinogen, and fXIII that activates the transglutaminase activity of factor XIII [25]. This interaction further strengthens the fibrin clot with covalent crosslinks, where fXIIIa introduces e-(γ-glutamyl)lysine cross-bridges between adjacent fibrin strands [25]. Therefore, since fXIII activity affects the overall 3-dimensional structure of the clot, we included the ΔcoaΔvwbp double mutant in our study. These findings indicate that coagulases, Coa and Vwb, contribute to the ability of *S. aureus* to generate robust clots.

Our recent work also demonstrated the contribution of coagulases in enhancing staphylococcal survival in fresh heparin-treated human blood and in WLM. Staphylococcal communities, in association with secreted coagulases, formed septic thrombi and were able to withstand high levels of stress inflicted by the host immune system and the administered antimicrobials (Manuscript 2). We observed that access to public goods conferred benefits onto cheats when they were cocultured with producers; these benefits were also observed for monocultures of producers. In contrast, monocultures of cheats alone did not exhibit enhanced survival in blood nor antibiotic tolerance in WLM (Manuscript 2). In addition to exploring the benefits of coagulases, we established that cheats were able to outcompete the producers in the WLM during scenarios where coagulation was unnecessary and cheating proved to be more advantageous; however, this fitness trend was abolished in experimental conditions where the benefits of coagulation outweighed cheating.

Cheats are well integrated and reside with producers within clots

Spatial organization is a key aspect within these formed clots because access to public goods often depends on how individuals localize themselves in relation to products of interest. We sought to visualize whether producers and cheats were able to coexist together within septic thrombi and used thin-sections of coagulated WLM that had been cocultured for 24 hours with LAC and Δcoa, or LAC and ΔcoaΔvwbp. Staining of WLM with concanavalin A (ConA) revealed co-aggregates of *S. aureus* enmeshed in a web of fibrin clots precipitated in the presence of coagulases (Fig. 1). Our micrographs show that producers and cheats are able to clump together and coexist indiscriminately within the in vitro septic thrombi, all the while being enclosed within the fibrous matrix (Fig 1.). This was true for both mutants, Δcoa and ΔcoaΔvwbp, that were cocultured with the LAC. Therefore, to reap the benefits of clots ensuing from secreted coagulases, the cheats situate themselves in close proximity to the producers where the
Figure 1. Intimate coaggregates of producers and cheats enmeshed in protective fibrin networks. WLM inoculated with 1x10^5 CFU USA300 LAC and its isogenic coa and vwbp variants. Cocultures of coagulated WLM were frozen in cryomatrix and subsequently sectioned. Sections were stained with Texas Red-conjugated ConA and DAPI and visualized by confocal fluorescence microscopy (with CFI Plan FL 40X oil objective [numerical aperture, 1.3]). Images are representative of stacks taken at 0.3 µm. The combination of cocultures are as follows: LAC gfp (green) & Δcoa (blue)(top row); LAC gfp (green) & ΔcoaΔvwbp (blue)(middle row); Δcoagfp (green) & LAC (blue) (bottom row). These panel images reveal intimate clusters of both producers and cheats coaggregating together, interspersed throughout the fibrous host matrix. There was no selection bias in the detection of the strains as demonstrated by the panels of the opposite combination in the bottom row.

the concentration of public goods is likely to be high. These observations prompted us to further investigate if coagulases are exploitable public goods and whether these social dynamics could be recapitulated in vivo.

Coagulases contribute to staphylococcal persistence resulting in organ injury and inflammation

Our murine model begins by challenging mice with an introduction of S. aureus into the host vasculature by injecting a sub-lethal suspension of 1x10^6 CFU S. aureus into their retro-orbital plexus. Our experiments examined the
consequences of variation for a single trait: the production of coagulases. In the monoinfection groups, mice were infected with only the LAC, \(\Delta_{\text{coa}}\), or \(\Delta_{\text{coa} \Delta_{\text{vwbp}}}\). Whereas, in the coinfection groups, mice were infected with a 1:1 mixture of both the LAC and \(\Delta_{\text{coa}}\), or LAC and \(\Delta_{\text{coa} \Delta_{\text{vwbp}}}\). Following injection into the retro-orbital plexus, the inoculum drains into the external jugular vein; this vein then converges in the thorax with the internal jugular and the subclavian veins into the ipsilateral superior vena cava [26]. Even though this route of delivery ensures high blood levels are achieved rapidly [27], the majority of inoculum disappears from the vasculature within six hours [28]. In addition to passive transport via blood-flow, the remaining staphylococci are able to survive within leukocytes and use them as a vehicle to escape the vasculature and taxi into uninfected regions of host tissues [29].

To assess the contribution of coagulases towards the hematogenous spread of \(S.\ aureus\) and establishment of metastatic infections, the kidneys, and spleens of animals were subjected to gross examination on the fifteenth day. Animal autopsies revealed that the kidneys from the monoinfection group harboring only producers (LAC) displayed signs of pyelonephritis, tissue ischemia and renal infarction (Fig. 2A and S2A). Similar pathology was observed for the coinfection groups harboring both producers and cheats (Fig. 2A and S2A). A high majority of these groups had visible abscesses protruding from the renal capsule for either one or both of the kidneys (Fig. 2D and S2A) In contrast, the majority of animals infected with only cheats (\(\Delta_{\text{coa}}\) or \(\Delta_{\text{coa} \Delta_{\text{vwbp}}}\)) had normal kidneys, displaying no ischemic tissue or abscesses (Fig. 2D and S2A). Thus, abscess lesions were not a result of default host responses to invading pathogens, indicating that the action of coagulases is required for staphylococcal persistence and sustained abscess lesions.

Spleens from the mono- and coinfection groups harboring producers (LAC) displayed signs of splenomegaly (Fig. 2B and S2B). Whereas the monoinfection groups harboring only cheats (\(\Delta_{\text{coa}}\) or \(\Delta_{\text{coa} \Delta_{\text{vwbp}}}\)) had normal spleens, displaying no signs of inflammation (Fig 2B and S2B). No abscesses were observed for the spleens in any of the infected groups (Fig. S2B). It should be noted that we observed corresponding pathology for both organs in each animal, where the inflamed spleen co-occurred with the abscessed kidneys (Fig. 2C). These data indicate that the inability of the host to clear the infection resulted in exaggerated inflammation in both organs and disease progression for the corresponding animals (Fig. 2C).

**Coagulases are critical public goods for staphylococcal abscess communities**

Staphylococcal dissemination into host tissue elicits a strong inflammatory response that attracts phagocytes such as polymorphonuclear neutrophils (PMNs) and macrophages to the site of infection to prevent microbial spread. Hence, in order for the indiscernible infectious foci to metamorphose into visible abscesses, \(S.\ aureus\) must escape phagocytic killing by PMNs, macrophages, and other immune cells. Therefore, to characterize the pathological consequences associated with coagulases and staphylococcal persistence, kidneys (representative of the majority within each group) of infected animals were removed on the fifteenth day, fixed in cryomatrix solution, thin-sectioned and subsequently processed for histological evaluation via hematoxylin-and-eosin (H&E) staining (Fig. 3).

The monoinfection group harboring only producers (LAC) developed disseminated abscess lesions (in a high majority of the kidneys) visible by light microscopy (Fig. 3A and S1A).
Swiss Webster mice were injected into the retro-orbital plexus with 1x10^6 CFU of USA300 LAC, Δcoa, or ΔcoaΔvwbp; as well as cocultures of LAC & Δcoa, or LAC & ΔcoaΔvwbp. On day 15, kidneys were removed from infected animals and examined during necropsy; (B) organs were imaged and weighed. (A) Quantification of pyelonephritis plotted as net weight of kidneys. (B) Quantification of splenomegaly plotted as net weight of spleens. (C) Animals with exaggerated inflammation of the spleen also had inflamed kidneys. The groups infected with LAC, LAC & Δcoa, or LAC & ΔcoaΔvwbp had kidneys (p<0.01) and spleens (p<0.001) that were significantly more inflamed than those infected with just Δcoa, or ΔcoaΔvwbp. (D) Quantification of renal injury and abscess formation plotted as the percentage of the kidneys with visible abscesses on the renal cortex; images represent the majority of the pathology for the kidneys of their respective group (* p<0.05, ** p<0.01 by analysis of ANOVA test). The image panels for all of the kidneys and spleens corresponding to these data can be found in Fig S1A and B.
Figure 3A.
Figure 3B.

Figure 3. **Coagulases are critical public goods that generate protective shields around staphylococcal abscess communities.** (A-B) Animals were injected into the retro-orbital plexus with $1 \times 10^6$ CFU of USA300 LAC, Δcoa, or ΔcoaΔvwbp; as well as cocultures of LAC & Δcoa, or LAC & ΔcoaΔvwbp. On day 15, kidneys were frozen in cryomatrix, thin-sectioned, stained with hematoxylin-eosin and histopathology images acquired by light microscopy and visualized with UPlan FL 20X, 40X oil objective, 100X oil objective. Visualization at 2X was carried out with Zeiss Stemi 2000-C stereomicroscope. Renal tissues of mice infected with LAC, LAC & Δcoa, or LAC & ΔcoaΔvwbp had visible abscess lesions (2X, red arrows). The staphylococcal abscess communities (100X) were enclosed by an amorphous eosinophilic pseudocapsule (20X and 40X, black arrow), surrounded by a zone of dead/necrotic leukocytes (20X, white box), and an outer zone of apparently healthy leukocytes (20X, green box). The renal sections of mice infected with only cheats, Δcoa or ΔcoaΔvwbp, were absent of any characteristic infectious lesions (2X), displaying healthy granular tissue (2X and 20X) and healthy leukocytes (40X, yellow arrow, and 100X).
The staphylococcal abscess communities were enclosed within an amorphous eosinophilic pseudocapsule circumscribed by zones of dead and necrotic PMNs. These hypercellular regions harbored a central nidus of bacteria, containing closely aggregated staphylococci (Fig. 3A and S1A). Similar abscess lesions and histology were observed for the two coinfections groups harboring both producers and cheats (Fig. 3B and S1B). These zones of infection and inflammation with mass accumulation of dead PMNs and staphylococcal aggregates were not visible for the monoinfection groups harboring only cheats (Δcoa or ΔcoaΔvwbp), exemplified by healthy renal tissue absent of abscess lesions (Fig. 3A and S1A).

A simple explanation for this observation is that by usurping the clotting cascade via secreted coagulases, S. aureus forms pseudocapsule barriers and fibrin deposits that prevent infiltrating immune cells from accessing the enclosed abscess communities. Hence, by co-aggregating with producers, this beneficial protection from coagulases is passively extended onto cheats as a form of bystander effect, ensuring their survival throughout disease progression. Taken together, these results indicate that coagulases generate shields to protect both producers and cheats in staphylococcal abscess communities and that the benefits of being shielded rely on the proximity of staphylococci to the ensuing coagulation product as observed in the WLM.

**Coagulases rescue cheats and enhance staphylococcal survival**

In order to verify that the access to public goods could rescue cheats from phagocytosis thereby allowing them to proliferate in host tissues, we further analyzed the beneficial contributions of coagulases by evaluating staphylococcal survival in the murine model. At day fifteen post intravascular injections, we harvested both the kidneys and spleen to determine the bacterial load for all animals. Comparing mice that had been infected with only the LAC, Δcoa, or ΔcoaΔvwbp, we found the bacterial load to be significantly higher for the LAC monoinfection group in comparison to the two monoinfection groups of only mutants (Fig. 4), where, Δcoa and ΔcoaΔvwbp were impaired in their ability to persist and replicate in host tissue.

![Figure 4. Access to coagulases reduces staphylococcal killing and enhances survival of cheats.](image)

Cohorts of 9 animals were injected into their retro-orbital plexus with 1x10^6 CFU of USA300 LAC, Δcoa, or ΔcoaΔvwbp; as well as cocultures of LAC & Δcoa, or LAC & ΔcoaΔvwbp. On day 15, (A) kidneys and (B) spleens were removed from infected mice, examined during necropsy and imaged (Fig. S1A and B); tissues were homogenized and plated on selective agar medium for enumeration of colony forming units. Data are representative of two independent trials (* p<0.05, ** p<0.01, *** p<0.001 by analysis of ANOVA test).

We then determined the consequence of coinfections on virulence, where coagulases were accessible to cheats. Animals were injected with 1:1 mixtures of producers and cheats into
their retro-orbital plexus. Using similar parameters, we compared mice that had been coinfectected with LAC and Δcoa, or LAC and ΔcoaΔvwbp. In contrast to the monoinfections, both Δcoa and ΔcoaΔvwbp displayed improved persistence in host tissue when present in coinfections with the LAC (Fig. 4). Additionally, we noticed a slight drop in the bacterial load for LAC when they were coinfectected with the mutants in comparison to when present alone. Taken together, these data indicate that coagulases are necessary for enhanced staphylococcal fitness and exaggerated pathology in host tissues. Additionally, cheats (Δcoa and ΔcoaΔvwbp) seemed to be able to exploit the public goods secreted by the producers (LAC) without paying the costs associated with synthesizing coagulases, thereby allowing them to increase in frequency. This trend held irrespective of whether we analyzed kidneys or the spleen for all of the groups. Hence, two of the hallmark benefits that define coagulases as public goods include enhanced virulence and bacterial fitness during infections.

Discussion

*Staphylococcus aureus* has certainly made a name for itself with its remarkable pathogenic potential, yet insights into the sociobiology of its pathogenesis have been lacking. A key aspect of this work was the identification of coagulases as essential public goods that can be exploited during *S. aureus* infections. We examined the contribution of these focal public goods by targeting Coa and vWbp, the hemostasis factors of *S. aureus* that allow it to take advantage of a system that is normally part of the host defense against bacterial pathogens. Coa and vWbp share significant secondary structural homology in their D1 and D2 domains that interact with ProT to form equimolar complexes that cleave fibrinogen to fibrin peptides. Despite both cofactors employing molecular sexuality mechanism to interact with ProT, their C-terminal halves are dissimilar, where vWbp includes a unique binding site for the blood glycoprotein, von Willebrand factor [18, 20, 30, 31]. In addition, vWbp displays lower binding affinity to ProT than Coa; the subsequent ProT-vWbp complex remains inactive until it undergoes a slow conformational change when its exosite binds to fibrinogen and generates fibrin fibrils at a reduced rate [20]. Perhaps this substrate-activated hysteretic kinetic mechanism explains the disparity in the coagulation of the WLM, where the Δvwbp mutant was able to generate clots within 24 hours, but Δcoa did not. Studies have suggested that vWbp is able to compensate for the lack of Coa during endocarditis [32, 33]; hence, another reason for the inclusion of the ΔcoaΔvwbp double mutant within our study.

An intravascular delivery route merits an address of certain gene products of *S. aureus* that contribute to its pathogenesis and survival in blood. *S. aureus*’ interaction with the physiological substrate, fibrinogen, is key for staphylococcal pathogenesis. Secreted factors Efb (extracellular fibrinogen binding protein), its homolog, Efb-h, and Eap (extracellular adherence protein) are known to bind to fibrinogen and/or fibrin, but they do so without generating clots [34-36]. Similar substrate affinity, independent of coagulation, is also attributed to the cell wall anchored surface proteins, ClfA and ClfB (clumping factors) [37-39]. ClfA and ClfB mutants display defects in survival in blood during endovascular and septic arthritis infections [40-44]; nevertheless, lack of either protein is not sufficient to abrogate abscess formation [28]. Even though the aforementioned factors interact with fibrinogen, disruption in either of these genes does not affect the ability of *S. aureus* to form fibrin clots, a phenotype attributed to the focal public goods of our study: coagulases. Therefore, we propose that the clotting of fibrin seems to be a critical event for establishing and sustaining staphylococcal abscess communities.
S. aureus dissemination into host tissues elicits swift proinflammatory responses mediated by the release of cytokines and chemokines, triggering a cascade of events that culminates in the activation of the adaptive immune response. Although not unique to our strains, pathogen-associated molecule patterns (PAMPs) play a significant role in alerting the host immune surveillance systems of invading staphylococci and, ultimately, coordinating massive leukocyte migration and phagocytic killing at the site of infection. Hence, S. aureus employs evasion mechanisms to prevent the infectious foci from being phagocytized in order to persist in host tissues. We demonstrate, by triggering ProT-mediated conversion of fibrinogen to fibrin, secreted coagulases generate barriers that hinder leukocyte infiltration into the staphylococcal abscess community. Histology micrographs revealed abscess lesions with staphylococcal aggregates surrounded by eosinophilic pseudocapsules (Fig. 3 and S1) in the kidneys of animals where S. aureus abscess communities were able to utilize the public goods. When coinfected with producers, cheats lacking coagulases were shielded and therefore able to survive and persist within the formed necropurulent foci (Fig. 3B, S2B and 4). Whereas, the absence of public goods in animals infected with only cheats, resulted in defects in abscess formation (Fig. 2D, 3A, and S1A) and staphylococcal survival (Fig. 4). Although, recruitment of inflammatory cells is a default host response to all invading pathogens, the presence of multiple abscess lesions we observed in the renal sinus (pelvis and calyces), renal medulla and cortex (parenchyma) (Fig. 3 and S1), indicates that staphylococcal persistence was dependent on coagulases. Furthermore, the inability of the innate mechanisms to eradicate staphylococci is further evidenced by the corresponding cases of splenomegaly in mice with abscessed kidneys (Fig. 2C). In addition to housing macrophages, dendritic cells, and lymphocytes, the spleen functions to filter antibody-complexed pathogens and mediate communication between corresponding cells of the innate and adaptive immune responses. This suggests that the increased cellularity of the spleens are due, in part, to the prolonged expansion and recruitment of phagocytes and leukocytes responding to persistent infection, attributed to the production of coagulases.

Even though we did not visually distinguish cheats from producers within the observed staphylococcal aggregates with our H&E stains, a dramatic improvement in their survival when coinfected with producers indicates that the abscess communities harbored both cheats and producers (Fig. 4). Indeed, the ability of S. aureus to clump together is modulated by the regulator systems, MgrA and ArlRS [45]. The fact that this trait is present in our strains, suggests that cheats and producers most likely clump together during the initial stages of infection, which can also be seen in the coagulated WLM where we observe intimate coaggregates of cheats and producers (Fig. 1). This is an important attribute, considering global phagocytosis in the infected area is also dependent on the size of the phagocytic targets, where clumps exceeding the upper size limit are unable to be ingested [46-48]. However, we deem coaggregation to be important because it places cheats in the immediate vicinity of the secreted public goods. Earlier work suggests that Coa is probably responsible for the formation of a fibrin shield directly around the bacterial cells, while vWbp induces fibrin formation closer to the abscess periphery [49, 50]. Therefore, by coaggregating with producers, cheats improve their survival by means of the neighboring coagulases that form a protective layer of fibrin around the central core of bacteria. Based on these observations we propose that the secreted coagulases are critical public goods and can, therefore, be exploited during infection in mixed populations.

S. aureus must meet its nutritional requirements to replicate in host tissues throughout disease
progression. For example, controlled acquisition of resources such as heme, zinc, and manganese is essential for staphylococcal survival in abscesses [51-53]. However, production of public goods is associated with fitness costs that divert resources away from primary metabolism. Hence, co-aggregation can lead to competition for resources between producers and cheats within staphylococcal abscess communities. Although not pronounced, the effects of competition and fitness costs are evident in our model. We observe a slight reduction in the number of producers when coinfected with cheats, in comparison to when present alone in monoinfections (Fig. 4). This is because monoinfection of LAC harbors only producers that cooperatively secrete coagulases; thus, the costs of producing public goods are shared. Whereas, in coinfections, which harbor both producers and cheats, the ∆coa or ∆coaΔvwbpS benefit from the coagulases secreted by the LAC without incurring the costs of producing the public goods themselves. These results suggest that cheating is advantageous in situations where costly public goods are readily accessible and cooperation isn’t enforced. This is perhaps why ‘natural’ cheats, coagulase-negative S. aureus, are able to emerge and evolve during clinical infections. Which begs the question: why are coagulases not policed?

Methods

Bacterial strains and growth conditions
Non-fluorescent versions of the strains were used in all experiments, except those involving confocal microscopy. Strains were grown in Tryptic soy broth (TSB) with 100 µg/mL rifampicin or streptomycin prior to inoculation of WLM or human blood. Enumeration of colony forming units was done on selective Tryptic soy agar (TSA) plates infused with 20 µg/mL rifampicin or streptomycin.

Generations of mutants and fluorescent tagging of strains
Please refer to Manuscript 2.

Screening coagulation phenotypes in WLM
The wound like medium (WLM) comprised of 45% Bolton broth, 50% bovine plasma, and 5% laked horse red blood cells. Glass 16 x 100 mm test tubes with caps were autoclaved, and 6 mL biofilm formulation media was aseptically dispensed into each tube. The overnight cultures were centrifuged for 10 min at 7000 rpm, washed and re-suspended in the same volume of 1x phosphate-buffered solution (PBS). The OD600 of all cultures was normalized. The glass tubes were then inoculated with 10 µL of 10⁴ to 10⁵ CFU/mL of S. aureus mono- or cocultures. The tubes are then incubated at 37°C for 24 h on an orbital rotator at 50 rpm.

Confocal laser scanning microscopy of coagulated WLM
WLM biofilms were prepared with a 460 µL volume being placed in a 5 cm x 0.5 cm glass tube and inoculated with 7.5 µL of 10⁴ to 10⁵ CFU/mL. Imaging was performed on frozen sample sections. Briefly, coagulated WLM was removed from tubes, placed in a Tissue-Tek compound to solidify. Frozen sections were transferred to Superfrost Plus microscope slides (Thermo Fisher Scientific) and stored at −20°C until ready for visualization. Frozen sample
sections were prepared for staining by air drying at room temperature for 5 min, washed three times in 1x PBS (2 min each time), fixed in 4% paraformaldehyde at room temperature for 15 min, washed three times in 1x PBS (2 min each time), allowed to air dry at room temperature for 5 min before addition of the stain. Matrix components were visualized by staining sections with 100 µg/mL Texas Red-conjugated concanavalin A (ConA) (Invitrogen, Carlsbad, CA, USA) in the dark for 5 min at room temperature, washing three times in 1x PBS (5 min each time), and then mounting with Prolong Gold Antifade reagent (Molecular Probes, Eugene, OR, USA) supplemented with 4', 6'-diamidino-2-phenylindole (DAPI) to stain DNA. Images were acquired with a Nikon T1-E microscope with A1 confocal and STORM super-resolution modules, and images were captured with an EMCCD camera (iXon Ultra 897, Andor, DU-897E-CS0-#BV) and analyzed with the NIS Elements program (version 4.50.00, Nikon, Japan).

Hematoxylin-and-cosin staining of renal sections
Approximately 8-10 kidneys were frozen in cryomatrix compound and thin-sectioned as described above, to a thickness of 6 and 12 µm. This independent set of frozen sections were subjected to hematoxylin-and-cosin (H&E) staining using standard laboratory techniques and were mounted with Permount mounting medium (Fisher Scientific) before visualization. Mounted slides were then imaged by light microscopy with an Eclipse 80i microscope (Nikon, Louisville, KY, USA), and images were captured with a DS-Fi1 camera (Nikon) equipped with a Digital Sight DS-U2 controller and analyzed with the NIS Elements program (version 4.51.01, Nikon, Japan).

Ethics statement
All animal experiments involved a protocol (#16046) that was reviewed, approved and performed under the regulatory supervision of the Institutional Animal Care and Use Committee (IACUC). Animals are managed by the Texas Tech University Laboratory Animal Research Center, which is accredited by the American Association for Laboratory Animal Science and National Institute of Health—Animal Research Advisory Committee (NIH ARAC). Animals are maintained in accordance with the applicable portions of the Animal Welfare Act and NIH ARAC guidelines. Veterinary care is provided under the direction of the full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine.

All animal procedures were performed by highly trained personnel. Animals were monitored for signs of morbidity and pain as specified by the IACUC: rapid respiration, slow, shallow or labored respiration; shock and ruffled fur; dehydration, inappetence and rapid weight loss; abnormal or hunched posture; animal not alert, abnormal movement; guarding reaction upon contact; vocalization when palpated or moved; self-mutilation, restlessness.
or lethargy. In addition, signs for judging infected animals to be moribund included any one of the following: complications associated with ocular pathology; impaired ambulation; evidence for muscle atrophy or emaciation. All animals were euthanized by sodium-pentobarbital injections, an approved method by the Panel on Euthanasia of the American Veterinary Medical Association.

**Acknowledgements**

We thank Sarah Julsrud for text revisions. Images were generated in the Image Analysis Core Facility supported in part by TTUHSC. This work was supported by the Danish Council for Independent Research grant 11106571.
References


Supplementary information

Supplementary Figure 1A.
Supplementary Figure 1B. Additional H&E images of kidneys representative of the majority within each group. (S1A-B) Animals were injected into the retro-orbital plexus with 1x10^6 CFU of USA300 LAC, Δcoa, or ΔcoaΔvwbp, as well as cocultures of LAC & Δcoa, or LAC & ΔcoaΔvwbp. On day 15, kidneys were frozen in cryomatrix, thin-sectioned, stained with hematoxylin-eosin and histopathology images acquired by light microscopy and visualized with UPlan FL 20X, 40X oil objective, 100X oil objective. Visualization at 2X was carried out with Zeiss Stermi 2000-C stereomicroscope. Renal tissues of mice infected with LAC, LAC & Δcoa, or LAC & ΔcoaΔvwbp had visible abscess lesions (2X, red arrows). The staphylococcal abscess communities (100X) were enclosed by an amorphous eosinophilic pseudocapsule (20X and 40X, black arrow), surrounded by a zone of dead/necrotic leukocytes (20X, white box), and an outer zone of apparently healthy leukocytes (20X, green box). The renal sections of mice infected with only cheats, Δcoa or ΔcoaΔvwbp, were absent of any characteristic infectious lesions (2X), displaying healthy granular tissue (2X and 20X) and health leukocytes (40X, yellow arrow, and 100X).
Supplementary Figure 2A.
Swiss Webster mice were injected into the retro-orbital plexus with $1 \times 10^6$ CFU of USA300 LAC, Δcoa, or ΔcoaΔvwbp, as well as cocultures of LAC & Δcoa, or LAC & ΔcoaΔvwbp. On day 15, kidneys and spleens were removed from infected animals and examined during necropsy. (A) Image panels of all the kidneys harvested from infected animals. The kidneys of animals infected with LAC, LAC & Δcoa, or LAC & ΔcoaΔvwbp had more abscesses and an overall worse pathology than those for animals infected with only Δcoa, or ΔcoaΔvwbp. (B) Image panels of all the spleens harvested from infected animals. The spleens of animals infected with LAC, LAC & Δcoa, or LAC & ΔcoaΔvwbp were much larger in size than those for animals infected with only Δcoa, or ΔcoaΔvwbp. White scale bars = 5mm.

**Supplementary Figure 2B.**

**Supplementary Figure 2.** Protection conferred by coagulases results in the inability of the host to clear the infection, resulting in (A) kidney abscesses and (B) exaggerated inflammation of the spleens.
Manuscript 4
Staphylococcus aureus coagulases rescue Pseudomonas aeruginosa during coinfection

Urvish Trivedi¹, Jonas S. Madsen¹, Cody Fell², Jake Everett³, Mette Burmølle¹, Kendra P. Rumbaugh², Søren J. Sørensen¹

¹Section of Microbiology, Department of Biology, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

²Department of Surgery, Texas Tech University Health Sciences Center, Lubbock Texas, United States of America

Abstract

Most infections harbor more than one microbe. Within such polymicrobial infections, microbes often produce extracellular factors that facilitate bacterial growth and virulence. While one member may produce these factors, their production can potentially benefit all constituent members within a local group or population. Staphylococcus aureus is a prominent member of polymicrobial infections where it secretes two clotting factors, staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp), that contribute towards staphylococcal survival and persistence during infections. As is the dilemma with many extracellular factors, we find that coagulases are also exploitable by bacteria that do not take part in producing them. In this study, we find that Pseudomonas aeruginosa, a Gram-negative bacteria, is able to benefit from the coagulases produced by S. aureus. P. aeruginosa displays enhanced survival in an in vitro clinical model and ex vivo samples of human blood where coagulases are available. Using a murine model of infection, we demonstrate that coagulases also contribute towards the hematogenous spread and persistence of P. aeruginosa in organ tissues. Our results provide a possible explanation as to why S. aureus and P. aeruginosa coinfections are recalcitrant and result in worse clinical outcomes.
**Introduction**

Polymicrobial infections are a significant and growing global health concern. The presence of multiple bacterial species is a common phenomenon in chronic wounds. Approximately 5 to 7 million Americans are treated for chronic wounds each year at an estimated annual cost of 20 billion dollars [1]. With an estimated rise in the prevalence of risk factors and associated costs of care over the coming years, the recent scope of interest shifts towards understanding the complex interactions that occur among the polymicrobial communities found in infections. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are among the most common organisms isolated from both acute and chronic wounds of various etiologies. It is well documented among clinicians that *S. aureus* is often the first bacteria to infect the wound and is subsequently joined by *P. aeruginosa* as the infection progresses. Owing in large part to their synergistic virulence against the host, *P. aeruginosa*–*S. aureus* coinfections are shown to be more severe and heal at a slower rate [2-4], and prove to be more recalcitrant to antimicrobial treatment than their monoculture counterparts [4, 5]. Even though the two are found together in human infections, *P. aeruginosa* has been shown to compete against and kill *S. aureus* when the two are cocultured together in standard liquid broth, *in vitro* cystic fibrosis model, and in the rat peritoneum [6-9]. In light of these facts, what are the benefits of these two pathogens coexisting in the face of competition?

A pathogen’s capability to attach to host tissue and produce extracellular virulence factors contributes to its ability to colonize and persist *in vitro*. However, in order to thrive during infection, pathogens must also utilize surrounding host extracellular matrix components (ECM) at the site of infection to evade the immune system and survive within the host. *S. aureus* is a Gram-positive facultative anaerobe that possesses an arsenal of adhesin/invasin gene products known to facilitate its interaction with the host ECM components during infection; among which lies a subgroup of proteins known to interact with plasma glycoproteins that are central players in the blood coagulation cascade such as prothrombin, fibrinogen, and fibrin. Staphylocoagulase (Coa) and von Willebrand factor binding protein (vWbp) are the two known coagulases of *S. aureus* that are able to bypass the physiological regulatory steps of blood coagulation pathways. The N-terminal residues of Coa and vWbp insert into the binding cleft in the prothrombin (*ProT*) catalytic domain, thereby activating the central coagulation zymogen in a non-proteolytic manner. The subsequent staphylothrombin complexes (*ProT*•Coa and *ProT*•vWbp) are then able to cleave fibrinogen and form insoluble polymerizing fibrin cables. The dense fibrous clot is further reinforced by the cross-linking activity of the transglutaminase factor XIII, a fibrin-stabilizing enzyme that is activated by vWbp [10].

The ability to generate clots is a defining feature of *S. aureus* infection, where coagulases can be regarded as public goods, whose production confers benefits onto all individuals present
within a local group or population. However, collaborative production of coagulases is not sustainable, where “free-riders” or non-producers do not cooperate in producing the public goods themselves but are still able to benefit from those secreted by the producers. Experiments using an in vitro infection and a murine abscess model established that coagulases are able to act as public goods in blood as well as staphylococcal abscess communities, where their contribution towards staphylococcal survival and pathogenesis was extended onto non-producers and not limited to only producers in a mixed community. The expression of these public goods was found to be a costly behavior that was vulnerable to being exploited by non-producers in situations where cooperation was not favored, resulting in a tragedy of the commons. This problem also applies in the fields of economics and human morality, where the tragedy is that – as a group – individuals stand to benefit from cooperation, but cooperation is not stable because each individual can gain by selfishly pursuing their own interests [11].

Our prior exploration of the social dynamics regarding coagulases was done on an intra-species level, where the degree of relatedness between the producers and non-producers was high; whereas, P. aeruginosa is a Gram-negative opportunistic pathogen whose genome is distant to that of S. aureus. Regardless, it remains a common member of polymicrobial infections, especially chronic wounds that harbor S. aureus. Therefore, whether or not coagulases can be exploited on an inter-species level remains unresolved. The goal of this work was to elucidate if P. aeruginosa could piggyback on and exploit this unique strategy S. aureus has evolved in usurping the hemostatic system for survival and replication during infection. Our model organisms include a community-associated methicillin-resistant S. aureus, USA300 LAC, that produces Coa and vWbp; an isogenic Δcoa mutant that does not produce Coa; and an isogenic ΔcoaΔvwbp double-mutant that does not produce Coa and vWbp. The isogenic variants, Δcoa and ΔcoaΔvwbp, represent non-producers that do not produce the public goods of interest, whereas LAC represents producers. We use PA14 wild-type as our model P. aeruginosa.

Results

Coagulases mediate clotting of wound-like media

Previous studies have established a convenient and reliable in vitro model for studying polymicrobial interactions [4, 6, 12, 13]. Termed ‘wound-like’ media (WLM), this model promotes the concomitant growth of P. aeruginosa and S. aureus for multiple days, a phenomenon otherwise unobservable in standard laboratory cultures [4]. The WLM incorporates physiological components of blood and is formulated to better simulate the nutrient conditions of wounds [12, 14, 15]. It is actively used in clinical practice to reconstitute the microbial populations from the wounds of individual patients by inoculating the WLM with debridement samples. Here we use the WLM to study the biofilm phenomenon most commonly associated with chronic wounds. The biofilm dogma has
historically considered the role of the bacterium-derived matrix. However, we pose the question: why must a pathogen form a self-secreted biofilm during infection, when surrounding host proteins can be modified and are available as building blocks?

Inoculation of fresh heparin-treated WLM with PA14 and LAC triggered clotting, whereas PA14 alone, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp were unable to generate clots within 24 hours (Fig. 1).

![PA14](image1.png) ![PA14 + LAC](image2.png) ![PA14 + Δcoa](image3.png) ![PA14 + ΔcoaΔvwbp](image4.png)

**Figure 1. Clotting of WLM is facilitated by Staphylococcus aureus coagulases.** Heparin-treated WLM was inoculated with PA14, as well as cocultures of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp and incubated for 24 hours at 37°C. Cultures were poured into a petri dish to assess coagulation. Data representative of three different determinations.

Even in the presence of circulating thrombin inhibitors (e.g. heparin) that effectively inhibit the clotting of the WLM and blood, the activity of staphylothermin complexes was not inhibited. Therefore the clotting of our WLM is attributed to the two known coagulases of *S. aureus*, Coa and vWbp; where *P. aeruginosa* is unable to catabolize fibrinogen to form fibrin clots.

**P. aeruginosa can coexist and situate itself in close proximity to the producers within clots**

At the macroscale, the clotted WLM provides a vast landscape in which the bacterial community can develop. However, in terms of access to public goods, spatial context is highly important, where it is valuable to consider interactions on the micron scale. Since coagulases are produced by *S. aureus*, their concentration is likely to be high in the vicinity of staphylococcal aggregates. It should be noted that here we regard coagulases as public goods that act on the surrounding host ECM in generating clots, and not directly on the surrounding bacterial cells. Therefore the benefits conferred onto *P. aeruginosa* from the ensuing coagulation product depend on how it situates itself in relation to the producers. To provide an accurate spatial context of our *in vitro* biofilm, we do not discriminate between the extracellular polymeric substance (EPS) and the host-derived matrix (HDM).

Thin-sections of coagulated WLM that had been cocultured for 24 hours with PA14 and LAC were stained and visualized using confocal laser scanning microscopy. Micrographs revealed clusters of PA14 and producers coaggregating indiscriminately, enmeshed in a web of fibrous matrix (Fig 2).
The coaggregates were interspersed throughout the fibrous HDM, all the while enclosed and segregated by fibrin strands. The HDM served as a scaffold to which the bacteria could adhere to and reside within. It should be reiterated that monocultures of PA14, and cocultures of PA14 and Δcoa, or PA14 and ΔcoaΔvwbp are unable to coagulate the WLM; therefore the observed fibrous architecture is orchestrated only in the presence of producers, where coagulases induce fibrinogen cleavage to polymerize protective layers of fibrin around the coaggregating clusters of cells. Taken together, these micrographs indicate that *P. aeruginosa* can inhabit the fibrin-enclosed pockets of the coagulated WLM generated at the expense of the producers. Our observations are in line with the biofilm phenomenon observed *in vivo*. There is evidence that bacteria infecting the wound site exist as aggregates, which form structured and spatially organized communities that are interspersed throughout the host tissue. Therefore, we decided to measure a classical parameter that is both dependent on community structure and biofilm formation: antimicrobial tolerance.
Access to coagulases confers enhanced antimicrobial tolerance

The clinical significance of biofilm-related infections is profound because they are more severe and recalcitrant to therapeutic intervention. Therefore, having visualized the marked phenotypic differences in the clotting ability of the WLM, we sought to determine if the phenomenon of enhanced antimicrobial tolerance was associated with our public goods of interest. Our experiments examined the consequences of variation for a single trait, whether or not PA14 had access to coagulases. In the monoculture group, the WLM was inoculated with only PA14. Whereas, in the coculture groups, WLM was inoculated with a 1:1 mixture of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp. The inoculated WLM were grown overnight, and antimicrobial tolerance was determined as described in Materials and Methods. We assessed their tolerance to an aminoglycoside (gentamicin) and an antimicrobial (ethanol). As shown in Fig. 3, cocultures of PA14 and producers (LAC) displayed higher tolerance to both gentamicin and ethanol in comparison to either cocultures of PA14 and non-producers (Δcoa and ΔcoaΔvwbp). The monocultures of PA14 and cocultures with non-producers displayed similar antimicrobial tolerance profiles.

Stressors can select for cooperation and defer a tragedy of the commons

Most chronic wounds display impaired rates of healing, taking months or years to do so, and can persist throughout an individual’s life. Over the course of their pathogenic life cycle, bacteria may be exposed to various treatments that can have a profound effect on their behavior and ultimately influence the population dynamics of the polymicrobial infection. Here we used the WLM to investigate the relative fitness of PA14 vs. producers, and PA14 vs. non-producers.

Our population dynamics experiment contained two treatment groups:
phosphate buffered solution (PBS) and gentamicin. The variation in our experiment is with respect to coagulation, which is the trait in whose evolution we are interested. We are able to focus on coagulases because the LAC wild-type, Δcoa, and ΔcoaΔvwbp mutants are initially identical at other parts of the genome, and so there is no genetic variation for other traits to influence selection. We varied PA14’s access to public goods by propagating them through multiple rounds of coculturing with LAC, to place them with producers, or with Δcoa or ΔcoaΔvwbp, to place them with non-producers. In the cocultures with producers, PA14 had access to coagulases, whereas in the cocultures with non-producers, they did not. However, in all cocultures PA14 had the potential to interact with staphylococci, allowing for global competition on an inter-species level. We initiated the experiment by inoculating the heparinized WLM with a 1:1 mixture of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp. After 24 hours of growth, the WLM was subjected to parallel treatments with either PBS (control) or gentamicin. Then, all subsequent rounds were initiated with the treated population from the preceding round. In order to calculate the relative frequency of P. aeruginosa vs. S. aureus, bacteria were enumerated after each round of treatment.

As we propagated the bacterial population through the different selection rounds, we found that PA14 was favored over producers under conditions of PBS treatment (Fig. 4A). However, non-producers increased in frequency in comparison to PA14 during the selection rounds of PBS treatment (Fig. 4A). This trend held irrespective of whether we analyzed the cocultures harboring Δcoa or ΔcoaΔvwbp. Therefore, the production of coagulases was not advantageous in conditions without stressors. This suggests that, since non-producers are not taxed with the costs of producing coagulases, they can divert resources towards other metabolic activities and maximize their growth rate, allowing them to better compete against P. aeruginosa. Alternatively, the spatial structure facilitated by coagulases may have reduced the growth rate of S. aureus.

In contrast, the producers were favored over PA14 under conditions of gentamicin treatment (Fig. 4B). Nonetheless, PA14 was able to survive in cocultures with producers. However, PA14 was completely eradicated after the third passage in cocultures with non-producers (Fig. 4B). When producers were the majority of the population, cooperation between producers is maintained, and the Coa and vWbp dependent fibrous matrix was sustainable. This is important because PA14 stands to benefit when the structural integrity of the coagulated WLM remains uncompromised. Whereas in cocultures with non-producers, neither the PA14, Δcoa, nor ΔcoaΔvwbp was able to clot the WLM, resulting in a fluid culture, leaving PA14 more susceptible to gentamicin. In addition, since PA14 did not clot the WLM, one can expect the fluid layers to harbor more PA14 than producers.
Figure 4. Consequential penalty of over-exploitation defers a tragedy of the commons.

Cocultures of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp were grown in heparin-treated WLM for 24 hours at 37°C. The population from these cultures were then subjected to PBS or gentamicin treatment and propagated through multiple days of culturing. Population ratios were assessed by enumeration of colony forming units on selective agar plates. Data represent three independent samples.

Therefore, PA14 is kept in check by the stressor and a tragedy of the commons is deferred, because exploitation of producers results in immediate consequential penalties. The WLM provides a powerful system for probing the role of bacterial interactions in vitro, in regards to antimicrobial tolerance and population dynamics of the microbial community; however, it lacks the immunological factors encountered during an infection in vivo. Therefore, we wanted to see if the benefits of secreted public goods could be recapitulated in actual wounds.

Coagulases are important for *P. aeruginosa* and *S. aureus* competitive fitness during hematogenous spread in a murine model

We examine these interactions in a more relevant vertebrate model of polymicrobial infection [16]. Although this is a nonlethal model, it provides a robust assay for examining the changes in composition of an infecting microbial community over several days. Therefore, we analyzed the contribution of coagulases towards bacterial survival in a murine chronic wound model. In these experiments, mice were administered a full-thickness surgical wound as described in Materials and Methods and were subsequently infected with a 1:1 mixture of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp. Their wounds were covered with transparent, semipermeable polyurethane dressing to protect the wound from contaminating bacteria. After 5 days of infection, the mice were euthanized, their wound tissue and spleen were harvested; and the bacterial load of *P. aeruginosa* and *S. aureus* in the tissue was determined.

Coinfections of the wounds resulted in comparable final growth yields for both strains in all of the groups, indicating that *P. aeruginosa* and *S. aureus* persisted in the wounds at similar levels (Fig. 5). However, the bacterial load in the spleen was higher for the mice coinfected with PA14 and LAC in comparison to the mice coinfected with PA14 and Δcoa, or PA14 and
Taken together, these results indicate that coagulases contribute towards the survival of *P. aeruginosa* and *S. aureus* during the hematogenous spread of infection. Suggesting that the effect on the *S. aureus* and *P. aeruginosa* numbers in the spleen is probably not due to increased persistence in wounds, but rather the ability of producers to usurp the coagulating cascade and form protective microthrombi as the bacteria seed into the host vasculature.

Therefore, to verify this, we decided to see if the benefits of secreted public goods could be recapitulated in fresh human blood.

### Coagulases enhance *P. aeruginosa* survival in human blood

The host complement system pairs with the antibody response of the adaptive immune system and provides an immediate defense against invading pathogens. Complement proteins are continuously synthesized by macrophages and hepatocytes and are abundant in blood serum. Complement proteins also form membrane attack complexes (MACs) that open destructive pores in the microbial cell envelope and cell wall [17-19]. In addition, these proteins can serve as markers that indicate the presence of a pathogen to phagocytes and enhance engulfment in a process called opsonization. Therefore, we analyzed the contribution of coagulases towards pathogen survival in fresh heparin-treated human blood. As before, the monoculture group was inoculated with only the PA14; whereas, the coculture groups were inoculated with a 1:1 mixture of both the PA14 and LAC; PA14 and Δcoa; or PA14 and ΔcoaΔvwbp. Monoculture of PA14 displayed a large reduction in CFU for *P. aeruginosa* in human blood (Fig. 6). In addition, cocultures of PA14 and Δcoa, and PA14 and ΔcoaΔvwbp suffered a large reduction in CFU, where both *P. aeruginosa* and *S. aureus* exhibited a defect in survival in human blood (Fig. 6). Interestingly, coculturing PA14 with LAC, significantly improved the survival of *P. aeruginosa* in human blood (Fig. 6). We also observed a lower reduction in the CFU of producers in

---

**Figure 5. Competitive indices of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp coinfections in a murine wound model.** Competitive index is defined as the log10 output ratio of PA14, LAC, Δcoa, or ΔcoaΔvwbp after 5 days of infection divided by the log10 input ratio of PA14, LAC, Δcoa, or ΔcoaΔvwbp, (A) represents competitive indices in splenic tissue and (B) represents competitive indices in wound tissue.
comparison to the non-producers for all cocultures. This trend was observed for both time points analyzed in our experiment. These data indicate that coagulases function as public goods and enhance pathogen survival during coculture in human blood, where Coa and vWbp generate fibrin shields that contribute towards masking complement factors and interfering with the phagocytic uptake of bacterial cells.

**Figure 6. Access to coagulases enhances P. aeruginosa survival in blood.** Monoculture of PA14 and cocultures of PA14 and LAC, PA14 and Δcoa, or PA14 and ΔcoaΔvwbp were incubated in heparin-treated human blood for 45 or 90 minutes and bacterial survival assessed by enumeration of colony forming units on selective agar plates. Data were generated from three independent trials. (* p<0.05, ** p<0.01 by analysis of ANOVA test)

**Discussion**

Although the importance of polymicrobial infections in clinical settings is clear, the paucity of studies examining the social dynamics of *S. aureus* and *P. aeruginosa* coinfections is largely due to the technical difficulties of growing the two pathogens together. Many of the studies elucidating the molecular details of their coexistence are carried out in liquid media and biofilm models that bear little or no clinical relevance. Thus models incorporating host ECM components that can better simulate *in vivo* nutrient conditions are crucial for understanding to what degree these polymicrobial interactions and mechanistic theories occur during infection. Coagulation is an ancient innate defense mechanism that traps and immobilizes invading pathogens in an effort to shield healthy tissue from the disseminating microbes [20-22]. This host response is not exclusive to *S. aureus* invasion, as it is also induced by chemical trauma, physical insult, and injection of sterile biological materials [23, 24]. *S. aureus* has developed a unique ability to co-opt the physiological coagulation cascade as part of its immune evasion and survival strategy. The goal of this research was to elucidate if the two known coagulases of *S. aureus*, Coa and vWbp, could serve as public goods during coinfection with *P. aeruginosa*.

Using relevant clinical models, our data reveal that *P. aeruginosa* is able to benefit from and exploit this unique trait to enhance its own survival and persistence during infection. The source of coagulases in these experiments was LAC, which during coculture produced public goods that potentiated survival of *P. aeruginosa*. During systemic coagulation, the staphylothrombin complexes interact with fibrinogen, an abundant host plasma protein that is a central player in the physiological coagulation pathways [25]. The staphylothrombin complexes cleave this 340-kDa soluble glycoprotein into fibrinopeptides that are subsequently rearranged to form thick insoluble fibrin cables that are cross-linked together to
form a clot. In addition to the producers being fortified, aggregates of *P. aeruginosa* are also incorporated into the polymerized fibrin meshwork (Fig. 2), where we find they are able to reap the protective benefits. *P. aeruginosa* residing in coagulated WLM with producers were significantly more tolerant to gentamicin and ethanol in comparison to when they were residing with non-producers (Fig. 3). The increased tolerance to antimicrobials here is attributed to the presence of fibrin deposits within the WLM biofilm.

The penetrating ability of gentamicin is dependent on the fundamental properties of electric charge, where the basophilic HDM poses a challenge for the positively charged antibiotic. Tseng et al. demonstrated that the positively charged antibiotic tobramycin became sequestered in the negatively charged biofilm periphery, while the neutral antibiotic ciprofloxacin readily penetrated them [26]. Therefore, the poor efficacy of gentamicin in our tolerance data was most likely due to its inability to access the fibrin-encased coaggregates. *P. aeruginosa* did display higher gentamicin tolerance when cocultured with *S. aureus* in comparison to when present in monocultures (Fig. 3), however this difference in tolerance was statistically insignificant.

Coinfections of *S. aureus* and *P. aeruginosa* result in greater virulence than monoinfections, therefore it is crucial to understand what parameters influence their coexistence and enhance their persistence *in vivo*. Binding of pathogen associated molecular patterns (PAMPs) to dedicated Toll-like receptors or nucleotide-binding oligomerization domain (Nod) proteins trigger specific signaling events that enhance the uptake and intracellular killing of invading pathogens to promote wound healing [27, 28]. Even though we do not see a difference in bacterial survival within the wounds of the mice, we demonstrate that access to coagulas does result in higher bacterial load within the splenic tissue (Fig. 5). The spleen functions to immunologically filter foreign substances and antibody-complexed pathogens from the blood, and this may provide a plausible explanation as to why we observe a higher bacterial load for the mice coinfected with producers and PA14 in comparison to those with non-producers and PA14. We surmise that the bacteria most likely seed from the wound into the host vasculature, where coagulases facilitate bacterial persistence and survival. Here we regard the product of coagulases’ activity, *S. aureus* and *P. aeruginosa* enmeshed within a fibrin meshwork, enables coaggregates to disseminate as thromboemboli and resist opsonophagocytic clearance by host immune cells. We further demonstrate the benefit conferred upon access to coagulases in human blood.

To survive in blood, bacteria must escape a variety of immune mechanisms, such as antimicrobial peptides, complement and phagocytic killing [29, 30]. Host immunity against bacteria typically involves complement proteins that recognize the microbial surface and promote killing via MACs. In addition, the entrapment and clearance of infection depend on the binding of complement proteins and antibodies, followed by pathogen recognition and the ability of phagocytes to access
P. aeruginosa displayed low survival rates in human blood when it was cultured by itself or when cocultured with non-producers (Fig. 6). However, P. aeruginosa survival improved dramatically upon being cocultured with producers that are able to generate clots (Fig. 6). Our observations align with other studies that have demonstrated that fibrin-encased bacteria do not activate immune cells and can thus escape phagocytosis [31, 32]. Earlier work suggests that Coa is probably responsible for the formation of a fibrin shield directly around the bacterial cells, while vWbp induces fibrin formation towards the periphery [33, 34]. We surmise that the fibrinogen and fibrin coat formed in the immediate vicinity, prevent attachment of complement proteins and antimicrobial peptides; whereas those formed distal to the coaggregates inhibit opsonin recognition by immune cells and act as a physical obstacle for incoming phagocytes. Together, these barriers generated by Coa and vWbp interfere with complement activation and opsonophagocytosis of the fibrin-encased bacteria.

The spatial distribution of different bacterial species in a biofilm provides important clues about the nature of their interspecies relationships. Visualization of the coagulated WLM revealed that even though the HDM is formed only in the presence of coagulases secreted by producers, aggregates of P. aeruginosa cells were also present and closely associated with the staphylococcal aggregates (Fig. 2). This phenomenon of coaggregation has also been observed in human biopsies, and murine chronic wound models [4, 6, 12]. The fact that P. aeruginosa coexists within and integrates itself within the HDM generated by producers is interesting because P. aeruginosa is shown to be highly lytic for S. aureus in well-mixed environments [35]. P. aeruginosa uses multiple small molecule signals to regulate its expression of virulence factors via quorum sensing (QS). Its ability to sense neighboring S. aureus results in elevated levels of quinolone signal-controlled toxins that enhances P. aeruginosa mediated killing of Gram-positive bacteria [35]. This shift proves detrimental to S. aureus growth rates and fitness, as it can lead to decreased ATP yield, and hemin or menadione autotrophy in S. aureus [36, 37]. However, this response is mitigated by the presence of the abundant serum protein, albumin, which binds and sequesters signaling molecules, thereby inhibiting the QS-regulated lytic pathways [13, 38]. This decrease in staphylococcal killing allows intimate coaggregates of P. aeruginosa and S. aureus to occur within our WLM similar to those observed in vivo. However, as a trade-off, coaggregation may lead to greater competition over resources.

A significant niche overlap between P. aeruginosa and S. aureus strains allows global competition to occur between the two species in the WLM. Consequently, in mixed populations where both LAC and PA14 are present, P. aeruginosa can gain the benefit of coagulase production without paying the cost and therefore increase in frequency, as they out-compete the cooperative S. aureus producer bacteria (Fig. 4A). This is because the cooperative production of public goods is associated with synthesis costs that divert resources away from
primary metabolism. Hence, co-aggregation can lead to competition for resources between producers and *P. aeruginosa*. Therefore, when there is a higher growth rate of one species within an individual culture, the bacteria from this culture will contribute a higher proportion of the bacteria chosen to initiate the next passage, and are (thus) more likely to be represented in the following round. This phenomenon is evident in our relative fitness assay, where *P. aeruginosa* did not incur the costs of secreting coagulases and therefore increase in frequency, as they out-compete the cooperative producers (Fig. 4A). However, such an advantage is bound to be short-lived as exploitation counteracts the communal benefits of public goods.

The structure of the fibrin network, rather than active communication and coordination among constituent members, is a key determinant for the observed population dynamics in the selection rounds with gentamicin. As stated above, the lytic antimicrobials are sequestered into the growing clots and affect the subsequent rate of lysis. Clots composed of a thick fibrous network with smaller pores are less permeable to antimicrobials and more resistant to lysis. As the overall community transitions from being in a well-established clot to a partially coagulated culture that is more fluid in nature, the weaker parts of the biofilm can harbor more *P. aeruginosa* than producers. Therefore, the bacteria within these weaker parts are eradicated/out-selected and thereby not passed onto the next round of culturing, allowing producers to retain majority and coagulation to be selected for. The *P. aeruginosa* that make it onto the following rounds are the ones that grow in intimate association with producers, where the concentration of public goods is likely to be high. Therefore, in the selection rounds of gentamicin treatments, a tragedy of the commons is deferred, where the expected payoff for *P. aeruginosa* is survival rather than outcompeting its benefactor (Fig. 4B). The instability of residing with only non-producers is further highlighted by a collapse of the *P. aeruginosa* community (Fig. 4B). These results demonstrate that public goods produced by *S. aureus* are beneficial even on an inter-species level and can be exploited during coinfection with *P. aeruginosa*. Understanding more about these interactions on a molecular scale will provide insight into how these co-infecting communities assemble and potentially into how to disrupt them, which is a necessary step forward in developing the next-generation of therapeutics.

**Methods**

**Bacterial strains and growth conditions**

Non-fluorescent versions of the strains were used in all experiments, except those involving confocal microscopy. Strains were grown in Lysogeny broth (LB) with or without 100 µg/mL rifampicin or streptomycin prior to inoculation of WLM or human blood. Enumeration of colony forming units was done on selective *Pseudomonas* isolation agar and/or *Staphylococcus* isolation agar.
Generations of mutants
Please refer to Manuscript 2.

Fluorescent tagging of strains
Please refer to Manuscript 5

Screening coagulation phenotypes in the WLM
The wound-like media (WLM) comprised of 45% Bolton broth, 50% bovine plasma, and 5% laked horse red blood cells. Glass 16 x 100 mm test tubes with caps were autoclaved, and 6 mL biofilm formulation media was aseptically dispensed into each tube. The overnight cultures were centrifuged for 10 min at 7000 rpm, washed and resuspended in the same volume of 1x phosphate-buffered solution (PBS). The OD600 of all cultures was normalized. The glass tubes were then inoculated with 10 µL of 10^4 to 10^5 CFU/mL of S. aureus mono- or cocultures. The tubes are then incubated at 37°C for 24 h on an orbital rotator at 50 rpm.

Confocal laser scanning microscopy of coagulated WLM
WLM biofilms were prepared with a 460 µL volume being placed in a 5 cm x 0.5 cm glass tube and inoculated with 7.5 µL of 10^4 to 10^5 CFU/mL of S. aureus mono- or cocultures. The tubes are then incubated at 37°C for 24 h on an orbital rotator at 50 rpm.

Relative fitness assay
WLM media was prepared as described above, and 460 µL of uncoagulated planktonic culture, or sections of coagulated WLM were suspended in 1 mL 1x PBS for 5 h, homogenized, vortexed, serially diluted and plated on selective TSA agar for enumeration of colony forming units. CFU was quantified per milliliter or per gram, respective to the samples. The 10^-2 dilution (10^4 – 10^5 CFU/mL) of the WLM homogenate was used to inoculate fresh WLM; this procedure was repeated every 24 h while propagating the bacteria through multiple days of culturing.
A similar setup was used for the gentamicin treated rounds, with the exception that the 460 µL of uncoagulated planktonic culture or sections of coagulated WLM were suspended in 300 µg/mL gentamicin for 5 h. Samples were then centrifuged and re-suspended in 1 mL of Dey-Engley broth, allowed to sit for 10 min. After which they were centrifuged and re-suspended in 1 mL of 1x PBS, homogenized, vortexed, serially diluted, and plated on selective *Staphylococcus* and *Pseudomonas* isolation agar for enumeration of colony forming units. CFU was quantified per milliliter or per gram, respective to the samples. The homogenate was used to inoculate fresh WLM for the following day; this procedure was repeated every 24 h, while propagating the bacteria through multiple days of culturing.

**Mouse wound model**

*S. aureus* and *P. aeruginosa* strains were grown overnight in fresh Lysogeny broth (LB) broth at 37°C. Bacteria were centrifuged at 7,500xg, washed, and suspended in the same volume of sterile 1xPBS. The OD₆₀₀ of all cultures was equalized. Six-week-old female Swiss Webster mice (Charles River Laboratories) were injected retro-orbitally with 1x10⁶ CFU (USA300 LAC) suspensions in 100 µL of PBS. On the fifteenth day post infection, mice were euthanized by sodium-pentobarbital injections and their wound beds and spleens excised. The tissues were weighed, and homogenized in sterile PBS. Serial dilutions of homogenates were used to determine CFU/g of tissue on *Staphylococcus* and *Pseudomonas* isolation agar.

**Blood survival assay**

Overnight cultures were centrifuged for 10 min at 7000 rpm and re-suspended in the same volume of 1x phosphate-buffered solution (PBS), repeated twice to rinse all antibiotics. Then the optical densities of all cultures were normalized for all of the strains to generate a suspension of 1x 10⁷ CFU/mL. Whole blood was collected from consenting human volunteer by venous puncture using a 0.6 x 19 mm x 305 mm and 23G x 3/4” x 12” push button blood collection set (Becton Dickinson); 4 mL of blood was collected into 13 x 75 mm vacutainers with 75 USP units of freeze-dried sodium heparin (Becton Dickinson). 450 µL of blood was aliquoted into a 1 mL Eppendorf tube and inoculated with 50 µL of bacteria sample (1x 10⁵ CFU/mL) from the monoculture or coculture suspensions. Samples were incubated at 37°C with slow rotation. 100 µL aliquots were removed at times 0, 45, and 90 minutes, mixed 1:1 with fresh 2% saponin/PBS and incubated on ice for 30 minutes. Five 1:10 serial dilutions were prepared and 100 µL aliquots spread on selective *Staphylococcus* and *Pseudomonas* isolation agar enumeration of colony forming units.

**Ethics statement**

The protocol for venous blood collection was approved by the Texas Tech University Health Sciences Center (TTUHSC) Institutional Review Board. Consent was obtained from healthy volunteers as mandated by the Clinical Research Institute (CRI) at TTUHSC in compliance with ethical practices. No admitted patients or children were involved in this study.
All animal experiments involved a protocol that was reviewed, approved and performed under the regulatory supervision of the Institutional Animal Care and Use Committee (IACUC). Animals are managed by the Texas Tech University Laboratory Animal Research Center, which is accredited by the American Association for Laboratory Animal Science and National Institute of Health—Animal Research Advisory Committee (NIH ARAC). Animals are maintained in accordance with the applicable portions of the Animal Welfare Act and NIH ARAC guidelines. Veterinary care is provided under the direction of the full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine.

All animal procedures were performed by highly trained personnel. Animals were monitored for signs of morbidity and pain as specified by the IACUC: rapid respiration, slow, shallow or labored respiration; shock and ruffled fur; dehydration, inappetence and rapid weight loss; abnormal or hunched posture; animal not alert, abnormal movement; guarding reaction upon contact; vocalization when palpated or moved; self-mutilation, restlessness or lethargy. In addition, signs for judging infected animals to be moribund included any one of the following: complications associated with ocular pathology; impaired ambulation; evidence for muscle atrophy or emaciation. All animals were euthanized by sodium-pentobarbital injections, an approved method by the Panel on Euthanasia of the American Veterinary Medical Association.

Acknowledgements
We thank Lauren Choate for technical assistance and Sarah Julsrud for text revisions. Images were generated in the Image Analysis Core Facility supported in part by TTUHSC. This work was supported by the Danish Council for Independent Research grant 11106571.
References


Manuscript 5
Should I stay or should I go? Adaptation in changing environments, selection for biofilm formation or motility

Urvish Trivedi¹, Maria R. Rebsdorf, Mette Burmølle¹, Søren J. Sørensen¹, Jonas S. Madsen¹

¹Section of Microbiology, Department of Biology, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

Abstract

The environment of most bacteria changes continuously and it is therefore key to understand how such changes affect bacterial evolution. Here, we studied which genetic and selective determinants promote phenotype specialization versus responsive switching during non-stable selection. This question is of major importance in concern with sessile (e.g. biofilms) and planktonic (e.g. swimming) phenotypes as they are fundamental to the fitness of bacteria. We evolved Pseudomonas aeruginosa PA14 in both constant and changing conditions where biofilm formation and/or swimming motility were selected for. In changing environments with global competition, a heterogeneous population evolved, where both specialists and generalists co-occurred. Interestingly, over time, generalists were outcompeted by the specialists; although, the generalists became better at both phenotypes compared to the ancestor. Further experiments revealed that generalists were more likely to prevail when local competition could occur subsequent to resettlement. Genome sequencing and measurements of cyclic di-GMP, a second messenger that facilitates the shift between biofilm-formation and swimming motility, revealed that mutations that affected this system were key for the adaptation of the phenotypes. Yet, neither specialists nor generalists lost their ability to regulate c-di-GMP levels. However, generalists had acquired mutations that optimized the bacterium’s phenotypic response to both types of environments by more efficiently shifting the intracellular level of cyclic di-GMP. We conclude that environmental change is critical for the adaptation of bacteria that ably shift between opposing physiological states; sessile and planktonic phenotypes. Yet, resettlement and local, as opposed to, global competition were key factors that stabilized the evolution of generalists.
Introduction

Niche specialization generally occurs in stable environments where selection favors organisms with the highest fitness. However, a high level of specialization for a specific niche typically makes the organism ill-adapted in alternative niches [1-3]. Most environments are heterogeneous and generalist organisms have therefore evolved to sense and respond to environmental change by switching their phenotype and thereby broadening their niche space. Responsive phenotypic switching can evolve if the environmental change is recurring, as the organism typically needs to sense the environmental change before expressing the phenotype that makes it most fit [4]. Organisms can also cope with environmental change by bet-hedging a strategy, which typically is an adaptation to unpredictable, sudden, and detrimental change. The type of phenotype, environmental change, and the frequency, therefore, influences what evolutionary strategy facilitates optimal fitness tradeoffs [5].

Here we study a central responsive phenotypic switch of bacteria, namely the transition between a sessile (e.g. biofilm formation) and a planktonic state (e.g. swimming). Biofilms are sessile communities of bacteria encased in a matrix composed of extracellular polymeric substances. Most bacteria are able to shift between such a sessile biofilm state and planktonic states, where the bacteria typically are singular and motile if proficient. Over the last couple of decades, research has led to a much better understanding of the biofilm state and the molecular adaptations that occur upon biofilm specialization [6-8], whereas the knowledge about adaptions to the planktonic motile state is more limited [9]. Yet, very little is known about how bacteria adapt towards responsive switching between the two states. Here, we studied the adaptation of this responsive phenotypic switch, using the biofilm model organism *Pseudomonas aeruginosa* PA14.

The environmental cues that promote each of the two opposing phenotypes are many and the regulatory machinery that controls and organizes the shift is complex. However, a second messenger, cyclic di-GMP (c-di-GMP), seems to be ubiquitously involved in facilitating this shift in the majority of bacteria, where high levels of c-di-GMP typically induce biofilm formation while low levels induce planktonic and motile states [10]. In *P. aeruginosa* the Wsp chemosensory system has been shown to be important for mediating the shift between the two states by controlling the intracellular level of c-di-GMP [11]. The Wsp system consists of seven proteins: WspA-F and WspR. WspA is a membrane-bound methyl-accepting chemoreceptor that responds to less well-defined external signal(s) [12, 13]. WspC is a methyltransferase and WspF is a methylesterase that regulates the activity of WspA by altering its methylation state. When hypermethylated, WspA activates WspR by phosphorylation. WspR is a diguanylate cyclase that upon activation synthesizes c-di-GMP, inducing the biofilm state.

Here, we performed experimental evolutions where we alternated between selection for biofilm formation and
swimming motility: Static broth cultures were used to select for biofilm formation as *P. aeruginosa* PA14 forms thick biofilm mats at the air-liquid interface, termed pellicles, under these conditions. Previous studies have shown that pellicle formation enables the bacteria to access oxygen, the energetically most favorable electron acceptor *P. aeruginosa* can utilize. Pellicle specialists can, therefore, outcompete non-specialists in static broth cultures [3, 14]. Swimming motility was selected for in low nutrient soft agar medium. Here a nutrient gradient is established by the actively metabolizing bacteria and swimming motility is triggered via chemotaxis. The further a bacterium can swim, the more access it has to nutrients, which is the basis of the selective pressure in the swimming assay [15]. The same basic nutrients were available in both assays. A number of generations were allowed to occur between each transfer as to allow adaptation strategies of responsive phenotypic switching, as opposed to bet-hedging, to occur in accordance with the aforementioned considerations.

**Results**

**Stable environments select for specialists with fitness tradeoffs in the opposing phenotype**

First, control experiments that mimicked stable environments by passing cultures from and to the same assay were examined. After a number of passes, the colony morphology and swimming phenotype of between 30 to 60 random isolates were tested. The colony morphology was assessed using a Congo red based assay, where a more wrinkled and red colony morphology is associated with elevated expression of the Pel matrix component and therefore correlated with increased biofilm formation [3]. Isolates with similar colony morphology and swimming phenotype (diameter) were assumed to be the same cell lineage (genotype). Fig. 1A (Fig. S1) illustrates how, compared to the ancestor (*A*0), the isolates from the biofilm (*C*<sup>b</sup>) and swimming (*C*<sup>s</sup>) control experiments had a more pronounced phenotype; more wrinkled and red colony morphology or larger swimming diameter, according to what phenotype had been selected for. The phenotypes of the isolates from these control experiments that were not selected for were consistently had less pronounced, suggesting a fitness tradeoff between the two phenotypes in question, in agreement with the physiological nature of the two phenotypes. This suggests that the biofilm and swimming control experiments selected for specialists.

An additional control experiment was performed, where cultures were passed from shaken culture to shaken culture. Here, the vast majority of isolates resembled the ancestral strain, confirming that the biofilm and swimming assays do indeed select for the phenotypes in question (Fig. 1A; *C*<sup>s</sup>) whereas shaken cultures did not.

**Changing environments select for heterogeneity**

When examining isolates from experiments where cultures were passed between the biofilm and swimming assays, mimicking changing environments, different lineages were observed. Compared with the control experiment it was clear that
environmental change selects for a more heterogeneous population (Fig. 1B). After changing the environment 11 times (e.g. 6 times in both the swimming and biofilm assay) both generalists and specialists were found, however, during selection for a longer period (35 rounds) the generalists were outcompeted (Fig. 1C). To further characterize the generalists and specialists, the competitive fitness of representatives of the lineages was found, relative to the ancestral strain, both in the biofilm and the swimming assay (Fig. 2). The competitive fitness of the generalists ($G^1$ and $G^2$) was enhanced compared to the ancestor in both the biofilm and swimming assay. Comparatively, the specialists had higher fitness in the assay that favored their phenotype and low fitness in the opposing assay. The average fitness (biofilm and swimming combined) was, however, similar amongst generalists and specialists. Yet, generalists were outcompeted over time during the experimental evolution (Fig. 1C).

**Early molecular adaption of specialists and generalists**

The genomes of one representative isolate from each of the unique lineages from experimental evolutions initiated with the original ancestor were genome sequenced to characterize the mutational adaptation of the strains (Table S1). All strains grown in changing environments had a mutation in $fleN$ ($FleN_{175C} >G$). This specific mutation leads to multi-flagellation and has previously been shown to increase swarming and swimming motility of *P. aeruginosa* PA14 [16]. The $fleN$ mutation was the only one identified in swimming specialist $S^i$.

**Figure 1. Environmental change facilitates phenotypic heterogeneity were generalists were outcompeted over time.** A) Examples of colony morphology and swimming motility of cell lineages. $A^*$, ancestral PA14; $C^B$, biofilm specialist from control experiments in the pellicle assay; $C^S$, swimming specialist from control experiments in the swimming assay; $C^G$, generalist from control experiments in shaken cultures; $G^i$, generalist from experimental evolution in changing environments. B) Number of different cell lineages observed in changing environments (Ch), shaken cultures (Sh), the pellicle assay (Pl), and swimming assay (Sw). White bars correspond to selection round 11, gray bars selection round 23, and black bars selection round 35. C) The proportion of generalists (green), biofilm specialist (red), and swimming specialist (blue) observed during experimental evolutions in changing environments over time.
Figure 2. Generalists with enhanced biofilm and swimming phenotypes were observed and fitness tradeoffs occurred among specialists. The competitive fitness of isolates of representative lineages measured in the swimming and the biofilm assay are shown relative to the ancestral strain A0. Data is based on 6 independent replicates and was Log2 transformed. Error bars represent the standard deviation.

Swimming specialists S2 had, in addition to the fleN mutation, a mutation in what was predicted as the ribosomal binding site upstream of groES. This mutation further increased the swimming ability of S2 compared to S1 but also made S2 less fit in the biofilm assay compared to the ancestor (Fig. 3). Missense mutations in swimming specialist S3 included the two aforementioned mutations in addition to a deletion mutation in PA14_29800 (PA14_29800_ΔRAG), which encodes a methyl-accepting chemotaxis protein (28% similar to WspA). The sensory module for signal transduction of PA14_29800 is different from the one WspA holds. Biofilm specialist B1 had, in addition to the fleN mutation, a mutation in wspA (WspA382V>A), a chemotaxis transducer of the Wsp system. Biofilm specialist B2 had, besides fleN and wspA, mutated in a lasR-like gene PA14_45960 (PA14_45960_ΔRAG). Mutations in generalists G1 and G2 were, in addition to that in fleN identified in wspF (WspF165Q>R) and wspA (WspA467F>C), respectively.

Based on the identified mutations of the isolates of the experimental evolutions in changing environments, two transitions seemed important for early adaptation of the generalist with higher competitive fitness than the original ancestor, namely: 1) The fleN (FleN175C>G) mutation enabled multi-flagellation, not only in swimming specialists but also in biofilm specialists (Fig. S2). Multi-flagellation propels the cells further, compared to the ancestor with a single flagellum, when motility
was induced. 2) Mutations in the Wsp chemosensory system, which are likely to affect the intracellular levels of c-di-GMP and thereby modify both swimming motility and the level of biofilm formation.

Multi-flagellation and modified cyclic di-GMP levels enabled specialization and enhanced responsive phenotypic switching of generalists

In accordance with our results, the specific mutation in $\text{FleN} \ (\text{FleN}_{175C>G})$ has previously been shown to infer a reduction in biofilm formation (Fig. 2; S1) [16]. We, therefore, speculated that the Wsp mutations in strain $G'$ and $G''$ compensated for this by amending the intracellular levels of c-di-GMP. Levels of c-di-GMP were therefore gauged by introducing a promoter-GFP fusion based c-di-GMP reporter into the different mutants. Fig. 3 shows the level of c-di-GMP of each strain relative to the ancestor ($A^0$) as a function of their competitive fitness against $A^0$ in the swimming and biofilm assays. Levels of c-di-GMP were positively correlated with the competitive fitness in the biofilm assay (Fig. 3; red symbols) and negatively correlated in the swimming assay (Fig. 3; blue symbols) revealing that the competitive fitness of the different strains was interrelated with levels of c-di-GMP. The c-di-GMP levels of biofilm specialists were considerably higher in the biofilm assay compared to the swimming specialist and the ancestor, however, their levels were also higher in the swimming assay. The poor swimming ability of biofilm specialists, the fitness tradeoff, was therefore caused by a lack of proficiency to reduce c-di-GMP levels enough to fully switch to the planktonic swimming state. In accordance, the opposite appeared to be the case for swimming specialists whose c-di-GMP levels were lower than those of the biofilm specialists and generalists in the swimming assay. It appeared that the c-di-GMP levels of swimming specialists were roughly the same as the ancestor in the biofilm assay, despite their lower fitness. Interestingly, the levels of c-di-GMP were also higher among generalist $G'$ and $G''$ in the biofilm assay, matching those of the biofilm specialists. However, $G'$ and $G''$ were apparently able to reduce c-di-GMP levels to below those of the biofilm specialists (Fig 3; stippled vertical line), roughly to the same levels as the ancestor. The span from low c-di-GMP levels in the swimming motility assay and the high levels in the biofilm assay was largest among the generalist, which indicates that the generalists adapted by bettering their c-di-GMP levels response, subsequent to becoming multi-flagellated.

Selection for generalists - resettlement, spatial isolation and local competition

The above suggested that a generalist strategy was not viable during global competition against both biofilm and swimming specialists. As motility is one of the phenotypes of interest, dispersal, and resettlement are relevant ecological factors to consider: Motility and other planktonic states enable bacteria to disperse and resettle in new environments and hereby become spatially isolated from competing lineages.
FIG 3. Cyclic di-GMP levels are interrelated with the competitive fitness of strains in both the swimming and biofilm assay. The competitive fitness of isolates as a function of c-di-GMP levels, both relative to the ancestral strain $A^0$. The stippled vertical line indicates were higher or lower level of c-di-GMP comes with fitness tradeoffs in the swimming and the biofilm assay, respectively. Cyclic di-GMP data is based on 4 independent replicates and was Log2 transformed. Error bars represent the standard deviation.

We mimicked resettlement by restarting the experimental evolutions in changing environments, beginning with the adapted generalists ($G^1$ and $G^2$) as the ancestors. The colony morphology and swimming performance of isolates were screened after 11 passes, corresponding to the first time point of the experimental evolutions started with the original ancestor (Fig. 1C). We found that the diversity was almost on par as in experiments initiated with the original $P. aeruginosa$ ancestor ($A^0$) both in experiments started with $G^1$ (Fig. 4A) and $G^2$ (data not shown), but much higher than the ones of the control experiments. Here, the ancestor lineages were, again, outcompeted by new lineages. However, in these experiments, the adapted lineages were not specialists that did very poorly in the opposing phenotype but were instead variations of generalists (Fig. 4B) with higher average fitness than the original ancestors $G^1$ and $G^2$. As can be seen on the y-axis of Fig. 4C, strains $G^1_{G1}$, $G^1_{G2}$, $G^1_{G3}$ and $G^1_{G4}$ outcompeted $G^1$ in the swimming assay but all except $G^1_{G4}$ had a slightly decreased competitive fitness in the biofilm assay compared to $G^1$. $G^1_{G4}$ did a little better in the biofilm assay compared to $G^1$ but was significantly less fit in the swimming assay compared to $G^1_{G1}$, $G^1_{G2}$, and $G^1_{G3}$. Gauging the c-di-GMP levels relative to those of the original ancestor ($A^0$) (Fig. 4C) showed that all derivatives of $G^1$ had a lower level of c-di-GMP compared to $G^1$ in the swimming assay.
In the biofilm assay the two most abundant strains; \( G_{G2} \) and \( G_{G3} \), had c-di-GMP levels comparable to \( G_{G1} \) while the levels of \( G_{G1} \) and \( G_{G4} \) were lower than \( G_{G1} \), yet higher than \( A_0 \). The above showed that after resettlement of \( G_{G1} \), selection favored generalist strains that adapted towards improved swimming motility while roughly retaining their biofilm phenotype.

As seen in Fig. 3, among the derivatives of \( G_{G1} \), levels of c-di-GMP were negatively correlated with the competitive fitness in the swimming assay (Fig. 4C; blue symbols). A positive correlation between the competitive fitness in the biofilm assay and c-di-GMP levels was less pronounced but was found (Fig. 4C; red symbols).

### Discussion

In accordance with the theory on adaptation in changing environments [1], our data indicate that during initial adaptation without spatial isolation, generalists will not be able to obtain as high fitness peaks in the two environments as the respective
specialists. We find that opposing phenotypes that normally cannot be expressed simultaneously by one individual, such as the biofilm and swimming phenotypes [10, 17] enhance the likelihood of a “jack of all trades, master of none” predicament in accordance with results based on modeling [18]. During the initial experiments, we found that specialists outcompeted generalists during global competition despite that the generalists gained a higher average fitness compared to the ancestor in the two environments. Yet, adaption to a higher level of responsive phenotypic switching, compared to the ancestor, did occur based on only a few mutations. These responsive generalists controlled their intracellular level of c-di-GMP more efficiently compared to the ancestor and the specialists. In accordance, further evolution of generalists after resettlement showed that the correlation between c-di-GMP levels and the biofilm and swimming phenotype (Fig. 4) was retained among these strains.

Mimicking relocation by restarting changing environment evolution experiments using the adapted generalists showed that specialists that lost the ability to perform opposing phenotype were selected against. This may suggest that specialists were less likely to evolve based on the genetic background of the adapted generalists (G' and G), but it is more likely that selection favored variations of generalists because the fitness peaks of the generalists in these experiments were closer to those possible to be obtained by the specialists. We speculate that fewer mutations can facilitate generalists compared to specialists, so the probability of generalists occurring would have been lower than specialists, supporting the above reasoning. Resettlement is a hallmark of the generalists [19] and facilitates spatial isolation. Resettlement, therefore, enables a shift from global, to more local competition against derivatives of the immediate ancestor [20]. Resettlement thus seemed to increase the probability of the successful evolution of generalists.

Bearing in mind that generalists during the experimental evolution were competing simultaneously with both biofilm and swimming specialists whose fitness was comparatively higher in the environment they’re specialized to (Fig. 2), suggests that a prerequisite for the success of specialists is that the number of dedicated specialists is equal to the number of possible niches, and that each specialist has a higher fitness peak in the relevant environment compared to a generalist. Importantly, this demonstrates that the establishment of one type of specialist (e.g. biofilm) depended on the other type of specialist (e.g. swimming) to efficiently outcompete the generalists during environmental change.

The shift between sessile and planktonic states are key to the fitness of bacteria and the complex regulatory systems that control this switch suggests that responsive phenotypic switching is a successful way of responding to environmental change as these sensing systems otherwise are too expensive to utilize and maintain in terms of energetics. Altering the levels of second messengers such as cyclic di-GMP has
been argued to be an energetically relatively cheap way to obtain plasticity [21]. We suggest that this may be why mutations that influence the c-di-GMP output were associated with adaptation towards increased responsive switching [22].

The fleN (FleN175C>G) mutation made S’ a better swimmer compared to the ancestor (A0) due to multi-flagellation. Interestingly, if fleN is knocked out, cells become multi-flagellated but immobile [16]. It is curious that the c-di-GMP level of S’ was lowered compared to A0 in the swimming assay but was similar in the biofilm assay where S’ was outcompeted by A’. This suggests that in order for the strains with the fleN mutation to become better at forming the biofilm, the c-di-GMP level needed to be additionally enhanced in the biofilm assay to compensate for the reduction that followed the fleN mutation. Missense (and non-nonsense) mutations in the Wsp system seemed to be a primary target for this. Here two outcomes were observed, either 1) mutations lead to a highly fit biofilm genotype that cannot reduce its c-di-GMP level sufficiently in the swimming assay and thus does poorly here, or 2) Wsp mutations that, despite facilitating high c-di-GMP levels in the biofilm assay, still allows the strain to reduce c-di-GMP levels sufficiently to also swim well. Data presented in Fig. 3 and 4C show that c-di-GMP levels of generalists and biofilm specialists in the biofilm assay reached approximately same heights. However in Fig. 3 swimming specialists had lower c-di-GMP levels in the swimming assay compared to generalists. This seemed to change after adaptation subsequent to resettlement, where the c-di-GMP levels of G’ derived strains were lower. Further adaptive specialization as a swimming specialist also seemed to influence systems that effect c-di-GMP levels, at least this may explain the difference in fitness between S’ and S’. It remains to be investigated how the mutation in the ribosomal binding site of groES furthered the swimming ability of the hyper-flagellated fleN mutated genotype.

“We plainly see that great benefit is derived from almost any change in the habits of life” [23].

Materials and Methods

Evolution experiments
Pseudomonas aeruginosa UCBPP-PA14 (A0) was used as the ancestral strain (A0) for evolution experiments other than the resettlement experiments where generalists G’ and G” were used as the ancestral strain. All experimental evolutions were performed in two independent replicates. Overnight cultures of ancestral strains were diluted to an OD600 of 0.15 and inoculated into the swimming and pellicle assay as described below. Changing environment Experimental evolutions in changing environments were started with the swimming assay and screening of the colony morphology and swimming motility were done after growth in the pellicle assay. Screenings were performed after 11, 23 and 35 passes in experiments started with A0, while screenings were done after 11 passes in experiments started with G’ and G”. Control experiments that mimicked stable environments were performed using A0 as the original inoculum and cultures were passed from and to the same assay. This was done in shaken
cultures, the pellicle assay, and the swimming assay. 35 passes were done for the control experiments. When screening colony morphology and swimming motility between 30 and 60 random colonies were isolated and re-streaked a minimum of 2 times.

**Colony morphology assay**

Ten microliters of overnight precultures (250rpm, 37 °C) adjusted to OD$_{600}$ 0.15 were spotted in the center of agar plates (94 x 16 mm) with 40 ml colony morphology assay medium; 10 g/l tryptone (Technova), 10 g/l agar (Technova), 40 ug/ml Congo red (CR), and 20 ug/ml Coomassie brilliant blue (CB). Colonies were grown at 24 °C for up to 5 days.

**Pellicle assays**

Two microliters of overnight precultures (250rpm, 37 °C) adjusted to OD$_{600}$ 0.15 were stabbed into the center of agar plates (94 x 16 mm) with 25 ml swimming agar; 5 g/l Miller LB broth (Microbiology), and 3 g/l agar (Technova). Agar plates were left at 37 °C for 16h. After incubation cells imbedded in the soft agar were transferred to sterile 50 ml Falcon tubes by use of a sterile spoon and homogenized by pipette mixing before either being passed or processed by flow cytometry.

Competitive fitness in pellicle assays: when competing two strains a gfp- and a mCherry-tagged version of both strains was used; strain A$_{gfp}$ was competed against B$_{mCherry}$ and A$_{mCherry}$ against B$_{gfp}$. Overnight precultures (250rpm, 37 °C) of all 4 strains were adjusted to OD$_{600} = 0.15$. The two sets of strains were mixed in 1:1 ratios and inoculated into the pellicle assay. Start ratios were measured and recorded by flow cytometry. After incubation, the ratio was again recorded by flow cytometry. CFUs were obtained for control experiments and the number of gfp and mCherry tagged bacteria were calculated based on identifying gfp-tagged CFUs using a Transillumination DR-88M.

**Swimming motility assays**

Two microliters of overnight precultures (250rpm, 37 °C) adjusted to OD$_{600}$ 0.15 were stabbed into the center of agar plates (94 x 16 mm) with 25 ml swimming agar; 5 g/l Miller LB broth (Microbiology), and 3 g/l agar (Technova). Agar plates were left at 37 °C for 16h. After incubation cells imbedded in the soft agar were transferred to sterile 50 ml Falcon tubes by use of a sterile spoon and homogenized by pipette mixing before either being passed or processed by flow cytometry.

Competitive fitness in swimming assays: when competing two strains a gfp- and a mCherry-tagged version of both strains was used; strain A$_{gfp}$ was competed against B$_{mCherry}$ and A$_{mCherry}$ against B$_{gfp}$. Overnight precultures (250rpm, 37 °C) of all 4 strains were adjusted to OD$_{600} = 0.15$. The two sets of strains were mixed in 1:1 ratios and inoculated into the swimming assay. Start ratios were measured and recorded by flow cytometry. After incubation, the ratio was again recorded by flow cytometry. CFUs were obtained for control experiments and the number of gfp and mCherry tagged bacteria were calculated based on identifying gfp-tagged CFUs using a Transillumination DR-88M.

**Insertion of gfp and mCherry into the chromosome of P. aeruginosa strains**

gfp or mCherry was inserted into the chromosome of P. aeruginosa strains by
using a mini-CTX2 based system [24]. More detailed procedures can be found in Almblad et al. [24] *P. aeruginosa* strains were made electrocompetent by washing (2 min. at 10000 g) 5 ml overnight cultures 3 times in 300nM glycerol. Cells were then pelleted, the supernatant removed, and resuspended in 50 µl 300nM glycerol. Next, plasmid mini-CTX2T2.1-GW::Ptrc-GFP or mini-CTX2T2.1-GW::Ptrc-mCherry was transformed into the strains. Transconjugants were selected for on LB agar supplemented with 100 ug/mL tetracycline. Hereafter pFLP2 [25] was used to remove the tetracycline resistance cassette following procedures described by [25], producing *gfp* or *mCherry* labeled, but antibiotic-sensitive *P. aeruginosa* strains.

**Genome sequencing and identification of mutations**

DNA was extracted from overnight cultures of *P. aeruginosa* strains using the Qiagen DNeasy Blood & Tissue kit (Qiagen, cat. no. 69504). The whole-genome sequencing libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, USA) and subsequently quantified by fragment analyzer™ (Advanced Analytical Technologies Co.). Sequencing was done as 2 x 250-base paired-end reads using the Illumina MiSeq platform (Illumina). Standard protocols were used for all of the above kits as provided by the manufacturer. Data were analyzed using CLC Genomic Workbench V7.5.1 (CLCBio). Obtained reads were trimmed and normalized. Briefly, reads were trimmed removing adapter sequences and discarding those of low quality using “trim sequences” tool (settings: ambiguous limit = 2, quality limit = 0.05). First, trimmed paired and orphan reads of the ancestor *A*, PA14 WT were mapped to the genome sequence of *Pseudomonas aeruginosa* UCBPP-PA14 (Accession number: NC_008463) using the “Map Reads to Reference” tool (default settings). Next, the same was done for trimmed paired and orphan reads of strains; *S*, *S*, *S*, *B*, *B*, *G*, & *G*. The lowest percentage of reads that matched was 99.9% (Table S2). Following this, a re-sequencing analysis was performed using the “basic variance detection” tool. The “probabilistic variant detection” tool (min. coverage 10 bp, variant probability 0.95) was then used to identify variations in the genomes of strains *S*, *S*, *S*, *B*, *B*, *G*, & *G* relative to the ancestral strain. The variance was verified or rejected by manually inspecting the individual mapped sequences. In addition, the trimmed paired and orphan reads were also assembled de novo using the “de novo assembly” tool (Min. contig length 500bp, Map reads back to contig; default settings). Reads were mapped back to contigs (min. length of contigs was set to 500 bp otherwise default settings). The de novo sequences were then inspected and mutations verified.

**Cyclic di-GMP reporter constructs**

Reporter pCdrA::gfp(ASV)§ [26] was introduced into the *P. aeruginosa* strains via electroporation and selected for on LB agar supplemented with 100 ug/mL gentamicin. Strains were made electrocompetent as described above.

**Flow cytometry**

Flow cytometry was performed on a FACSArie IIIu (Becton Dickinson Biosciences, San Jose, CA, USA).
Settings and voltages; forward scatter = 505V, side scatter = 308V, green fluorescence (bandpass filter 530/30 nm) = 508V, and red fluorescence (bandpass filter 610/20 nm) = 500V. A 70 um nozzle was used at a sheath fluid pressure of 70 psi. The BD FACSDiva software v.6.1.3 was used for operating and analyzing results.

Competition assays: gates were defined using gfp and mCherry tagged and non-tagged versions of the P. aeruginosa PA14 wild-type (A0). When obtaining ratios of green and red fluorescent P. aeruginosa, only fluorescent events were counted and they were only counted if the events were not both green and red. Gates and quantifications were verified by CFU counting.

Cyclic di-GMP measurements: a gfp threshold was defined using a non-tagged version of P. aeruginosa UCBPP-PA14 (A0) and the same strain carrying the pCdrA::gfp(ASV)S reporter. Cells from shaken cultures, the pellicle of static cultures, and the swimming assay were used when setting the threshold. Samples were taken at different distances from the edge of the advancing swimming zone down to the center of the swimming zone. The pCdrA::gfp(ASV)S reporter is based on the expression of an unstable version of GFP and the number of green events that exceeded the threshold was used as a measure of population averaged c-di-GMP levels.

Calculating relative competitive fitness and relative community c-di-GMP levels
The relative competitive fitness of strains was calculated as ratios of gfp and mCherry events (# of mutants divided by # of ancestors) when biofilm and swimming assays were incubated (start ratio) and after incubation (end ratio) and this number was then log2 transformed. E.g., Relative competitive fitness = log2([start ratio]/[end ratio]). The relative community c-di-GMP levels were calculated by log2 transforming the number of gfp events of the derived strain divided by those of the ancestor (always the A0 strain).

Imaging using transmission electron microscopy
Transmission electron microscopy was performed directly from overnight cultures. 400 mesh carbon-coated Cu/Rh grids were glow discharged for 30 seconds immediately prior to use. Grids were incubated on 10 µL of overnight culture for 1 minute before blotting the liquid media off with filter paper. Subsequently, each grid was incubated in 10 µL of 1% uranyl acetate for 2 minutes before blotting. Finally, each grid was blotted with 10 µL of ddH2O, and blotted dry with filter paper. Grids were left to dry before image acquisition. Images were acquired using a CM 100 transmission electron microscope and captured using an Olympus Veleta camera. All acquired photos were used to assess the distribution of the number of flagella by manual counts.

Acknowledgements
We would like to thank Dr. Henrik Almblad and Prof. Tim Tolker-Nielsen very much for sharing their beautifully constructed vectors CTX2T2.1-GW::Ptrc-GFP, mini-CTX2T2.1-GW::Ptrc-mCherry and pCdrA::gfp(ASV)S. Also, a big thanks...
you to Anette Hørdum Løth and Luma George Odish for excellent help with laboratory experiments. This work was supported by the Danish Council for Independent Research grant 11106571.
References


FIG S1A. After 11 passes in changing environments

A^0 0%

S^2 33%

S^1 15% 52% Swimming specialists

S^A 4%

G^A 11% 11% Generalists

B^1 37% 37% Biofilm specialists
FIG S1B. After 23 passes in changing environments

- A\(^0\): 0%
- S\(^3\): 45%
- S\(^6\): 10%
- S\(^c\): 3%
- G\(^1\): 2.5%
- G\(^2\): 2.5%
- B\(^{1-}\)-like: 20%
- B\(^2\): 17%

- 58% Swimming specialists
- 5% Generalists
- 37% Biofilm specialists
FIG S1C. After 35 passes in changing environments

A^0  0%

S^D  26%

S^C-like  14%

S^B-like  12%  60% Swimming specialists

S^E  5%

S^F  3%

B^1-like  3%  40% Biofilm specialists

B^2-like  36%

FIG S1D. After 11 passes in swimming assays

A^0  0%

C^S1  90%  100% Swimming specialists

C^S2  10%
FIG S1E. After 12 passes in pellicle assays

A^0  0%
C^{B1}  60%
C^{B2}  40%

100% Biofilm specialists

FIG S1F. After 23 passes in shaken cultures

A^0  100%
C^{sh}  100%

100% Generalists
Table S1. Identified mutations based on full genome sequencing

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pass #</th>
<th>Mutation</th>
<th>Affected protein</th>
<th>Mutation nt</th>
<th>Mutation aa</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>G \textsuperscript{1}</td>
<td>23</td>
<td>SNP\textsubscript{1}</td>
<td>FleN (PA14_45640)</td>
<td>4059726 A&gt;C</td>
<td>175 C&gt;G</td>
<td>cd02038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNP\textsubscript{2}</td>
<td>WspA (PA14_16430)</td>
<td>1406990 T&gt;G</td>
<td>449 F&gt;C</td>
<td>cd11386</td>
</tr>
<tr>
<td>G \textsuperscript{1}</td>
<td>23</td>
<td>SNP\textsubscript{3}</td>
<td>FleN (PA14_45640)</td>
<td>4059726 A&gt;C</td>
<td>175 C&gt;G</td>
<td>cd02038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNP\textsubscript{4}</td>
<td>WspE (PA14_16480)</td>
<td>1412596 A&gt;G</td>
<td>185 Q&gt;R</td>
<td>cd11386</td>
</tr>
<tr>
<td>S \textsuperscript{1}</td>
<td>11</td>
<td>SNP\textsubscript{1}</td>
<td>FleN (PA14_45640)</td>
<td>4059726 A&gt;C</td>
<td>175 C&gt;G</td>
<td>cd02038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNP\textsubscript{5}</td>
<td>RBS of groES (PA14_57020)</td>
<td>5081023 A&gt;C</td>
<td>185 Q&gt;R</td>
<td>cd02038</td>
</tr>
<tr>
<td>S \textsuperscript{1}</td>
<td>11</td>
<td>SNP\textsubscript{6}</td>
<td>PA14_32850</td>
<td>4059726 A&gt;C</td>
<td>175 C&gt;G</td>
<td>cd02038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNP\textsubscript{7}</td>
<td>PA14_45960</td>
<td>4085368 C&gt;T</td>
<td>231 A&gt;T</td>
<td>cd02038</td>
</tr>
</tbody>
</table>

Colors highlight similar mutations in different strains.


Table S2. Mapping summary report

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total reads after trimming</th>
<th>Number of reads match</th>
<th>Average coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A \textsuperscript{1}</td>
<td>1271202</td>
<td>1269398</td>
<td>x52</td>
</tr>
<tr>
<td>G \textsuperscript{1}</td>
<td>2518088</td>
<td>2515557</td>
<td>x70</td>
</tr>
<tr>
<td>G \textsuperscript{2}</td>
<td>3810762</td>
<td>3807083</td>
<td>x103</td>
</tr>
<tr>
<td>S \textsuperscript{1}</td>
<td>5180430</td>
<td>5174869</td>
<td>x140</td>
</tr>
<tr>
<td>S \textsuperscript{2}</td>
<td>3572136</td>
<td>3568627</td>
<td>x116</td>
</tr>
<tr>
<td>S \textsuperscript{3}</td>
<td>4762343</td>
<td>4758130</td>
<td>x133</td>
</tr>
<tr>
<td>B \textsuperscript{1}</td>
<td>3552979</td>
<td>3549348</td>
<td>x96</td>
</tr>
<tr>
<td>B \textsuperscript{2}</td>
<td>3010215</td>
<td>3007620</td>
<td>x83</td>
</tr>
</tbody>
</table>

Reads were mapped against the complete genome of *Pseudomonas aeruginosa* UCBPP-PA14 (Accession number: NC_008463)
FIG S2. TEM pictures of selected strains
Murine burn wound infections were performed with adult female Swiss Webster mice. Animals were infected with $10^2$ CFU of *P. aeruginosa* WT, Swimming specialist (S3), Biofilm specialist (B2), Generalist (G1), Generalist (G2), or Mixed populations. Animals were monitored for signs of morbidity or mortality over the course of 7 days and the survival rates for each group was recorded. Experiment was performed at twice with 5 animals per experimental group. The group infected with *P. aeruginosa* WT displayed the highest mortality rate, followed by the Swimming specialist (S3). The group infected with the Generalist (G1) and (G2) had somewhat similar survival rates. This was also true for the Mixed population groups; it should be noted that the two mixed population groups had identical survival rates. The Generalist (G1) and (G2), and Mixed population groups displayed higher survival rates than those of the *P. aeruginosa* WT and Swimming specialist (S3).
Murine burn wound experiment was performed as described in Fig. S4. This data corresponds to the animals of the burn wound survival experiment of Fig. S4. Autopsies were carried out for each animal at the time of death. Their spleen, tissue distal to the burn wound, and the center of the burn wound were excised, homogenized and assessed for bacterial load by enumeration of colony forming units on agar plates. All infected animals had nearly similar bacterial loads for the center and distal sites of the burn wound. However, the animals infected with the Biofilm specialist (B2) had much lower bacterial load in their spleen in comparison to all other groups. Suggesting that the high expression levels of c-di-GMP affected their ability to spread systemically during acute infections.

**FIG S4.** High expression levels of c-di-GMP results in lower systemic spread. Murine burn wound experiment was performed as described in Fig. S4. This data corresponds to the animals of the burn wound survival experiment of Fig. S4. Autopsies were carried out for each animal at the time of death. Their spleen, tissue distal to the burn wound, and the center of the burn wound were excised, homogenized and assessed for bacterial load by enumeration of colony forming units on agar plates. All infected animals had nearly similar bacterial loads for the center and distal sites of the burn wound. However, the animals infected with the Biofilm specialist (B2) had much lower bacterial load in their spleen in comparison to all other groups. Suggesting that the high expression levels of c-di-GMP affected their ability to spread systemically during acute infections.
FIG S5. Competitive indices of *P. aeruginosa* WT, Swimming specialist (S^3^), and Biofilm specialist (B^2^). The murine burn wound assay was performed as described in Fig. S4, with the exception of the experiment being terminated after 24 h of post infection. This was done to determine the systemic spread of the two specialists displaying opposite virulence patterns, independent of a variation in time. Thus all strains were given the equal amount of time to spread systemically. After 24 h autopsies were carried out for each animal at the time of death. Their spleen, tissue distal to the burn wound, and the, center of the burn wound were excised, homogenized and assessed for bacterial load by enumeration of colony forming units on agar plates. As the data demonstrates, each animal harbors almost similar number of bacteria within the burn wound and sites distal to it. However, as demonstrated, the fitness in systemic spread requires the ability to tune down the levels of c-di-GMP. The mutations in the flagellar motility apparatus could also potentially influence their ability to spread systemically, however, a mutation in this apparatus was present in all strains and not just the Swimming specialist. Therefore, we believe the optimization of c-di-GMP expression to play a greater role and perhaps be the determining factor for the mortality rates observed in acute burn wounds.
Appendix
Staphylocoagulase, an exploitable intra- and inter-specific public good

Urvish Trivedi, Jonas S Madsen, Mette Burmølle, Søren J Sørensen
Section of Microbiology, University of Copenhagen, Denmark

Introduction

Staphylococcus aureus and Pseudomonas aeruginosa are a major cause of community-acquired infections and one of the leading causes of nosocomial infections. The ability of these pathogens to colonize their hosts depends upon the cooperative production of extracellular factors. Here we study the role of the staphylocoagulase (Coa) in the formation of microcolony-associated fibrin structures. Given that this phenotype is important during infections, we used an experimental evolution approach by culturing these pathogens together in a clinically relevant in vitro wound model.

Aim of the study

i. Is coa vital in developing a bacterium-derived matrix?

ii. Are there fitness costs associated with coa production?

iii. Is it a trait that is exploitable on a both intra- and inter-specific level?

Phenotype screening for thrombus formation in MRSA

Experiments were started using three bacterial strains: a wild-type Staphylococcus aureus probe and a coa negative mutant, and a wild-type P. aeruginosa coa- mutant, and a wild-type coa+ producing individuals. The relative fitness of each strain was monitored while propagating the bacterial populations through several rounds of culturing.

Population dynamics experimental design

Experiments were started using three bacterial strains: a wild-type S. aureus that produces coa, a coa mutant, and a wild-type P. aeruginosa. The relative fitness of each strain was monitored while propagating the bacterial populations through several rounds of culturing.

Relative fitness

Costs and benefits of cooperation in respect to population dynamics and community structure within S. aureus and P. aeruginosa biofilms

Figure 1. Trajectory of the coagulase and coa.

Figure 2. Ability to produce coa and form a thrombus enhances antibiotic tolerance in S. aureus. A variant strain expressing higher antibiotic tolerance was compared to a non-resistant strain grown aerobically overnight in 2% CO2 in culture tubes. The antibiotic and susceptibility tolerance of variants from these cultures were then measured. Error bars represent the standard error of the means. All results are shown as colony forming units per gram for three replicates per experimental condition.

Figure 3. Conditions favouring coa production delvers a tragedy of the commons. Coa production was measured in co-cultures propagated without treatment, antibiotic treatment, and PAO1 coa::Tn. Error bars represent the standard error of the means. All results are shown as colony forming units per gram for three replicates per experimental condition.

Figure 4. Conditions favouring coa production delvers a tragedy of the commons in mixed inter-species, but not in intra-species, biofilms.

Figure 5. P. aeruginosa displays higher antibiotic tolerance when co-cultured with coagulase positive S. aureus. P. aeruginosa mono-cultures and S. aureus coagulase negative mutants were grown aerobically overnight at 37°C in culture tubes. The antibiotic and susceptibility tolerance of variants from these cultures were then measured. Error bars represent the standard error of the means. All results are shown as colony forming units per gram for three replicates per experimental condition.
Conference Presentations during PhD fellowship (oral lectures)


Trivedi U., Madsen J.S., Burmølle M., Sørensen S.J. (2015) Staphylocoagulase, an exploitable intra- and inter-specific public good. Danish Society of Microbiology Annual Conference, Copenhagen, Denmark

Conference Presentations during PhD fellowship (Posters)


Trivedi U., Madsen J.S., Burmølle M., Sørensen S.J. (2015) Staphylocoagulase, an exploitable intra- and inter-specific public good. Danish Society of Microbiology Annual Conference, Copenhagen, Denmark

Scientific Grants/Awards during PhD fellowship

Villum Foundation Grant for Confocal Bio-imaging Microscope (2016)
European Campus of Excellence PhD Summer School Scholarship (2015)
Co-authorship Statements
3A. Co-authorship statement
All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper

<table>
<thead>
<tr>
<th>1. PhD student</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name:</strong> Urvish Trivedi</td>
</tr>
<tr>
<td><strong>Department:</strong> Biology, Section of Microbiology</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Paper/Manuscript</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Title:</strong> A post-planktonic era of in vitro infectious models: issues and changes addressed by a clinically relevant wound like media.</td>
</tr>
<tr>
<td><strong>Authors(s):</strong> Urvish Trivedi, Jonas S. Madsen, Kendra P. Rumbaugh, Randall D. Wolcott, Mette Burmølle, Søren J. Sørensen</td>
</tr>
<tr>
<td><strong>Journal:</strong> Critical Reviews in Microbiology</td>
</tr>
<tr>
<td><strong>Vol/page:</strong> 43/453–465</td>
</tr>
<tr>
<td><strong>DOI:</strong> <a href="http://dx.doi.org/10.1080/1040841X.2016.1252312">http://dx.doi.org/10.1080/1040841X.2016.1252312</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. Contributions to the paper/manuscript made by the PhD student</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>What was the role of the PhD student in designing the study?</strong></td>
</tr>
<tr>
<td>The PhD student conceptualized the review topic and focus of the paper writing in this study.</td>
</tr>
<tr>
<td><strong>How did the PhD student participate in data collection and/or development of theory?</strong></td>
</tr>
<tr>
<td>The student initiated the writing of the review and discussed the topics needing to be addressed. Contacted appropriate collaborators for their expert opinions and reviews.</td>
</tr>
<tr>
<td><strong>Which part of the manuscript did the PhD student write or contribute to?</strong></td>
</tr>
<tr>
<td>The student wrote all parts of the manuscript, and incorporated suggestions of collaborators.</td>
</tr>
<tr>
<td><strong>Did the PhD student read and comment on the final manuscript?</strong></td>
</tr>
<tr>
<td>Yes.</td>
</tr>
</tbody>
</table>
4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)?

Please indicate which degree/thesis:

Please indicate which specific part(s) of the paper that has been produced as part of the PhD study:

---

Signatures

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

Date: 01.12.2017  Name: Søren Johannes Sørensen  Signature:

Date: 30-11-17  Name: Urvish Trivedi  Signature:

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but not included in the PhD thesis. You can find your PhD secretary here: www.science.ku.dk/phd/

Revised March 2016
# Co-authorship declaration

**All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper.**

## PhD student

<table>
<thead>
<tr>
<th>Name</th>
<th>Urvish Trivedi</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCPH user id:</td>
<td>jdw666</td>
</tr>
<tr>
<td>Department:</td>
<td>Biology, Section of Microbiology</td>
</tr>
</tbody>
</table>

## Paper/Manuscript

**This co-authorship declaration applies to the following:**

- **Title:** The sociobiology of coagulases, two distinct public goods of Staphylococcus aureus in a clinically relevant in vitro infection model.
- **Authors(s):** Urvish Trivedi, Jonas S. Madsen, Jake Everett, Cody Fell, Jakob Haaber, Alexander Horswill, Mette Burmølle, Kendra P. Rumbaugh, Søren J. Sørensen
- **Journal:**
- **Vol/page:**
- **DOI:**

## Contributions to the paper/manuscript made by the PhD student

1. **What was the role of the PhD student in designing the study?**
   - The PhD student performed the majority of design, data collection, analysis and paper writing in this study.

2. **How did the PhD student participate in data collection and/or development of theory?**
   - The student designed the study and assays used in this manuscript, conducted imaging and microscopic imaging, analysis of images and data.

3. **Which part of the manuscript did the PhD student write or contribute to?**
   - The student wrote all parts of the manuscript, and incorporated suggestions of collaborators.

4. **Did the PhD student read and comment on the final manuscript?**
   - Yes.
4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)?  

Yes

Please indicate which degree/thesis:

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

Signatures

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

Date: 30-11-17  Name: Søren Johannes Sørensen  Signature: 

Date: 30-11-17  Name: Urvish Trivedi  Signature: 

Date: Name:  Signature: 

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but not included in the PhD thesis. You can find your PhD secretary here: www.science.ku.dk/phd/
3A. Co-authorship statement

All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper.

<table>
<thead>
<tr>
<th>1. PhD student</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name: Urvish Trivedi</td>
</tr>
<tr>
<td>Department: Biology, Section of Microbiology</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Paper/Manuscript</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title: What’s mine is yours: the essential and yet exploitable nature of coagulases during Staphylococcus aureus infection</td>
</tr>
<tr>
<td>Authors(s): Urvish Trivedi, Cody Fell, Jonas S. Madsen, Jake Everett, Mette Burmølle, Kendra P. Rumbaugh, Søren J. Sørensen</td>
</tr>
<tr>
<td>Journal:</td>
</tr>
<tr>
<td>Vol/page:</td>
</tr>
<tr>
<td>DOI:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. Contributions to the paper/manuscript made by the PhD student</th>
</tr>
</thead>
<tbody>
<tr>
<td>What was the role of the PhD student in designing the study?</td>
</tr>
<tr>
<td>The PhD student performed the majority of design, data collection, analysis and paper writing in this study.</td>
</tr>
<tr>
<td>How did the PhD student participate in data collection and/or development of theory?</td>
</tr>
<tr>
<td>The student designed the study and assays used in this manuscript, conducted imaging and microscopic imaging, animal experiments, clinical modelling, analysis of images and data.</td>
</tr>
<tr>
<td>Which part of the manuscript did the PhD student write or contribute to?</td>
</tr>
<tr>
<td>The student wrote all parts of the manuscript, and incorporated suggestions of collaborators.</td>
</tr>
<tr>
<td>Did the PhD student read and comment on the final manuscript?</td>
</tr>
<tr>
<td>Yes.</td>
</tr>
</tbody>
</table>
4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)? [ ] Yes

Please indicate which degree/thesis:

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

---

**Signatures**

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

<table>
<thead>
<tr>
<th>Date</th>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>01.12.2017</td>
<td>Søren Johannes Sørensen</td>
<td>![Signature]</td>
</tr>
<tr>
<td>30-11-17</td>
<td>Urvish Trivedi</td>
<td>![Signature]</td>
</tr>
<tr>
<td></td>
<td>Name:</td>
<td>Signature:</td>
</tr>
</tbody>
</table>

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but **not included** in the PhD thesis. You can find your PhD secretary here: [www.science.ku.dk/phd/](http://www.science.ku.dk/phd/)
3A. Co-authorship statement
All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper.

1. PhD student
Name: Urvish Trivedi  
UCPH user id: jdw666
Department: Biology, Section of Microbiology

2. Paper/Manuscript
This co-authorship declaration applies to the following:
Title: Staphylococcus aureus coagulases rescue Pseudomonas aeruginosa during coinfection
Authors(s): Urvish Trivedi, Jonas S. Madsen, Cody Fell, Jake Everett, Mette Burmølle, Kendra P. Rumbaugh, Søren J. Sørensen
Journal:
Vol/page:
DOI:

3. Contributions to the paper/manuscript made by the PhD student
What was the role of the PhD student in designing the study?
The PhD student performed the majority of design, data collection, analysis and paper writing in this study.

How did the PhD student participate in data collection and/or development of theory?
The student designed the study and assays used in this manuscript, conducted imaging and microscopic imaging, animal experiments, clinical modelling, analysis of images and data.

Which part of the manuscript did the PhD student write or contribute to?
The student wrote all parts of the manuscript, and incorporated suggestions of collaborators.

Did the PhD student read and comment on the final manuscript?
Yes.
4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master’s degree)?

| Yes | No |

Please indicate which degree/thesis:

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

---

**Signatures**

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

<table>
<thead>
<tr>
<th>Date</th>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>01.12.2017</td>
<td>Søren Johannes Sørensen</td>
<td></td>
</tr>
<tr>
<td>30-11-17</td>
<td>Urvish Trivedi</td>
<td></td>
</tr>
</tbody>
</table>

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but *not included* in the PhD thesis. You can find your PhD secretary here: [www.science.ku.dk/phd/](http://www.science.ku.dk/phd/)

Revised March 2016
3A. Co-authorship statement
All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student’s contribution to the paper.

1. PhD student
Name: Urvish Trivedi  
UCPH user id: jdw666
Department: Biology, Section of Microbiology

2. Paper/Manuscript
This co-authorship declaration applies to the following:
Title: Should I stay or should I go? Adaptation in changing environments, selection for biofilm formation or motility
Authors(s): Urvish Trivedi, Maria R. Rebsdorf, Mette Burmølle, Søren J. Sørensen, Jonas S. Madsen
Journal:
Vol/page:
DOI:

3. Contributions to the paper/manuscript made by the PhD student
What was the role of the PhD student in designing the study?
The PhD student helped with the design, data collection, and paper writing in this study.

How did the PhD student participate in data collection and/or development of theory?
The student helped design the study and assays, conducted imaging and microscopic imaging, and generating the data.

Which part of the manuscript did the PhD student write or contribute to?
The student helped write the manuscript with large influence from the primary investigator.

Did the PhD student read and comment on the final manuscript?
Yes.

Revised March 2016
4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)?

Yes

Please indicate which degree/thesis:

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

<table>
<thead>
<tr>
<th>Date</th>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-11-17</td>
<td>Jonas S. Madsen</td>
<td></td>
</tr>
<tr>
<td>30-11-17</td>
<td>Urvish Trivedi</td>
<td></td>
</tr>
</tbody>
</table>

Signatures

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but not included in the PhD thesis. You can find your PhD secretary here: [www.science.ku.dk/phd/](http://www.science.ku.dk/phd/)

Revised March 2016
Social dynamics & adaptive strategies of microbes

PhD Thesis

Urvish Trivedi

Department of Biology
Section of Microbiology