Investigating New Roles for Septins in Host-Pathogen Interactions Using *Staphylococcus aureus*

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Declaration

I, Stevens Eugene Robertin, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Septins are eukaryotic cytoskeletal proteins involved in numerous cellular processes including host-pathogen interactions. During infection by the Gram-negative pathogen *Shigella flexneri*, bacteria are entrapped in septin cages for host defense. Previous work suggested that septins associate with the Gram-positive pathogen *Staphylococcus aureus,* but determinants of these interactions remained unknown. To address this, we investigated septin-*S. aureus* interactions by using infection of human epithelial HeLa cells and our cell-free *in vitro* reconstitution platform based on purified septin complexes.

S. aureus invasion is a crucial step to successfully establish infection. In **Chapter 3** we discovered that septins are recruited with actin to *S. aureus* engaging the receptor integrin α5β1 during host cell invasion. We also showed that septin depletion affected bacterial adhesion and invasion, as well as total protein levels of integrin α5β1 and focal adhesion kinase.

Following invasion, intracellular *S. aureus* must evade host defense mechanisms. In **Chapter 4** we used HeLa cells expressing a cytosolic *S. aureus* escape marker to investigate septin recruitment to cytosolic or vacuolar bacteria, and tested a wide range of experimental parameters on septin-*S. aureus* interactions.

In **Chapter 5,** our cell-free *in vitro* system revealed that septins can directly bind the *S. aureus* surface. We also showed that wall teichoic acids (WTA), the major autolysin Atl and Sortase A restricted septin binding *in vitro* and during HeLa cell infection. WTAs and Atl are known to restrict peptidoglycan recognition by host immune sensors. We performed peptidoglycan pulldown assays and showed that purified septins bind to purified *S. aureus* peptidoglycan, highlighting a novel role for septins as a sensor of peptidoglycan.

Overall, our findings identified new ways to block *S. aureus* invasion that may also apply to other pathogens engaging integrin α5β1 for cellular invasion, and further characterisation of peptidoglycan sensing by septins may inspire new host-directed antibacterial strategies.

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As a first-generation PhD student, I want to underscore my appreciation for my parents, they've dedicated themselves to supporting my education to the best of their abilities and resources. A decade ago, I embarked on my research career as an intern working on the microbiome of coral reefs in Guadeloupe. Back then, I could never have imagined that this path would lead me to explore such different scientific areas in different countries and ultimately become a doctor myself. This PhD journey has been an extraordinary rollercoaster of emotions, and, as I reach the conclusion of this chapter of my life, I am very excited for what lies ahead.

Sé grènn diri ki ka fè sak diri, sa ki la pou'w dlo paka chayé'y.

Yékri, Yékra !

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List of abbreviations

ADP : adenosine diphosphate

ATP : adenosine triphosphate

Agr : accessory gene regulator

Arp2/3 : Actin Related Protein 2/3 complex

CW : Cell wall

CWT : Cell wall targeting domain

DAPI : 4',6-diamidino-2-phenylindole

F-actin : Filamentous actin

FA : Focal adhesion

Fn : Fibronectin

FnBPA/B : Fibronectin-binding proteins A and B

G-actin : Globular actin

GBP : Guanylate-binding protein

GFP : Green fluorescent protein

GST : Glutathione S-transferase

GTP : guanosine triphosphate

GTPase : guanosine triphosphate phosphatase

LC3 : Microtubule-associated protein 1A/1B-light chain 3

LTA : Lipoteichoic acids

LPS : Lipopolysaccharide

MOI : Multiplicity of infection

Mprf : Multiple peptide resistance factor

MRSA : Methicillin-resistant *S. aureus*

MSCRAMMs : Microbial surface components recognizing adhesive matrix molecules

MSSA : Methicillin-sensitive *S. aureus*

msGFP : Monomeric superfolder green fluorescent protein

NPPC : Non-professional phagocytic cell

PG : Peptidoglycan

PGRP : Peptidoglycan recognition protein

P62/SQSTM1 : sequestosome 1

SEPT : Septin

siRNA : small interfering RNA

SrtA : Sortase A

WT : Wild-type

WTA : Wall Teichoic Acids

Chapter 1. Introduction

As the poet Maya Angelou said, "You can't use up creativity. The more you use, the more you have" (Angelou, 1982). Echoing the creativity displayed by individuals facing adversity, bacterial pathogens developed innovative strategies in their coevolutionary arms race with their hosts to subvert their cellular machinery. Benefiting from the use of a variety of bacterial pathogens over the course of four decades, research in the field of "cellular microbiology" has illuminated key aspects of the host cytoskeleton hijacked by bacteria to invade cells and replicate intracellularly (Isberg and Falkow, 1985; Cossart et al., 1996; Haglund and Welch, 2011; Robertin and Mostowy, 2020).

This chapter serves to introduce key concepts and set the stage for our investigation. Firstly, we will introduce the cytoskeleton, highlighting how complex interactions between its different components are key for essential cellular processes. Then we will demonstrate how in recent years, septins — viewed as the fourth component of the cytoskeleton — emerged as a central player regulating different microbial pathogens. Specifically, the observation that septins associate with the major human pathogen Staphylococcus aureus has provided inspiration for this thesis, which aims to investigate the role of septins during S. aureus infection. The final section of this introduction chapter will overview S. aureus virulence and its interactions with host cells, providing insights on how studying the infection of host cells by S. aureus can help to unveil new roles for the septin cytoskeleton in host-pathogen interactions.

1.1. The cytoskeleton

The cytoskeleton is an intricate network of filamentous proteins that provide mechanical support and allows for cell shape change during processes such as cell movement and division (Fletcher and Mullins, 2010). The term "cytosquelette" (cytoskeleton in French) was coined in 1931 and extensive research over the years has led to identification of different cytoskeletal proteins and their functions (**Figure 1)** (Wintrebert, 1931; Hohmann and Dehghani, 2019). The cytoskeleton was originally considered to consist of three major components, including actin filaments, microtubules and intermediate filaments. Septins, a highly conserved family of guanosine triphosphatase (GTPases), are increasingly recognised as a fourth component of the host cytoskeleton associating with cell membranes, actin filaments and microtubules (Mostowy and Cossart, 2012).

1.1.1. Actin

Actin monomers (also called globular actin or G-actin) polymerise to form actin filaments (filamentous actin or F-actin) (**Figure 1A**) which are thin, double-helical filaments that are typically 7-9 nanometers in diameter (Dominguez and Holmes, 2011). Actin filaments are polar, with a fast-growing end (+) where addition of monomers generally occur, and a slow-growing end (-) where disassembly mainly occurs. Among actin nucleators, the Actin Related Protein 2/3 complex (Arp2/3) and the Diaphanous-related formin-1 (mDia1) catalyse the initial formation of filament "seeds" to generate new filaments where required. The activity of nucleators is promoted by Rho-guanosine 5'-triphosphatase (Rho-GTPase) and nucleation-promoting factors (NPFs) such as SH3 Protein Interacting with Nck (SPIN90) which synergise the action of Arp2/3 and mDia1.

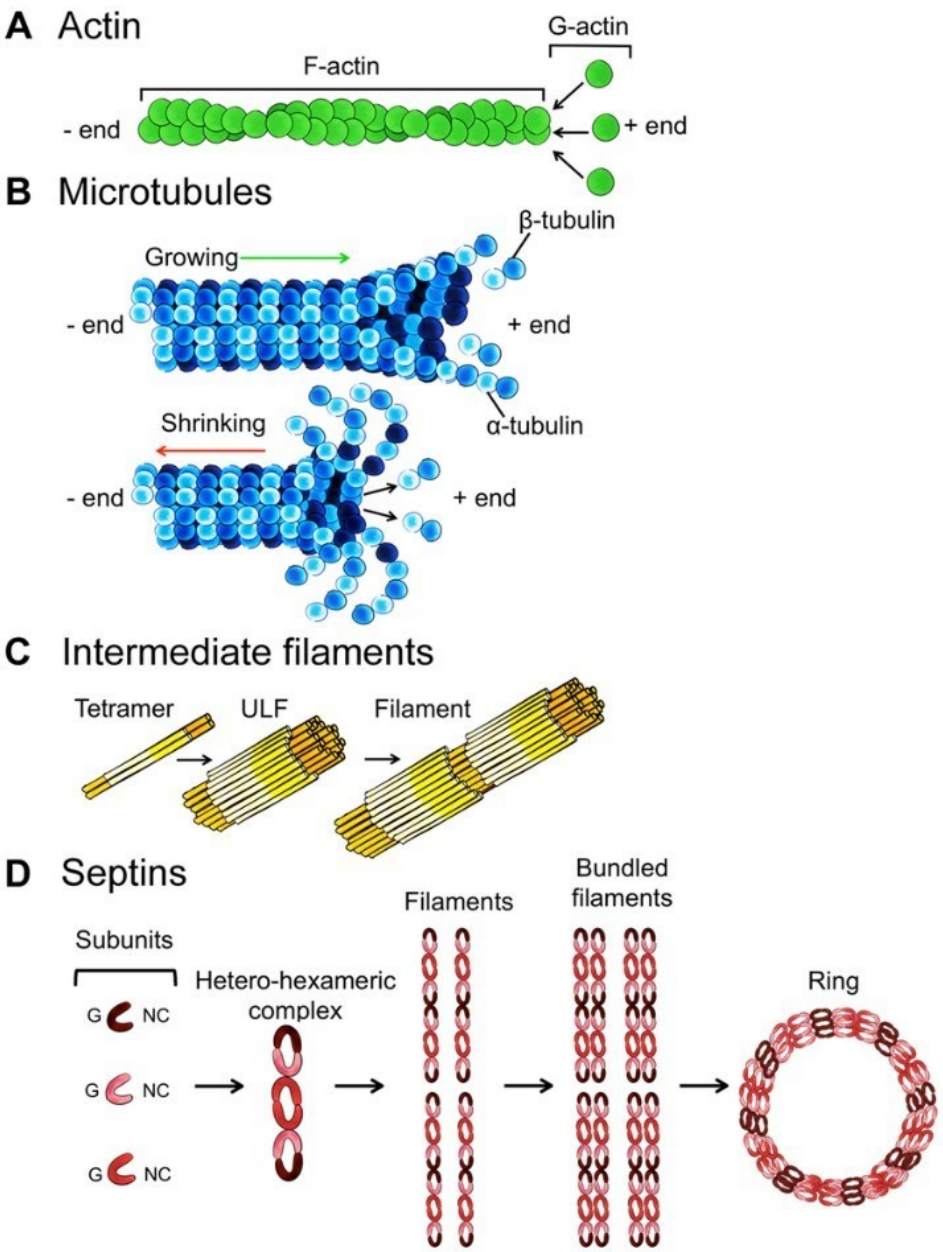

Figure 1. The eukaryotic cytoskeleton. The eukaryotic cytoskeleton is consisting of actin filaments, microtubules, intermediates filaments and septins. **A**. G-actin polymerize to form F-actin growing by the addition of monomers to the plus end preferentially. **B**. αβ-tubulin heterodimers assemble into microtubules and disassemble in a process called dynamic instability. **C**. The initial step in intermediate filaments assembly is formation of homodimer or heterodimer pairs of IF proteins, which then assemble into symmetric tetramers that will form an intermediate state called the unit length filament (ULF) before assembling into mature filaments. **D**. Core septin subunits can assemble into hetero-hexameric complexes to form higher-order structures such as filaments, bundles and rings. Adapted from (Mostowy, 2014).

(Cao *et al.*, 2020). Additionally, the activities of nucleators are controlled by actin binding proteins such as profilin which promote filament elongation by increasing the rate of exchange of actin-bound ADP for ATP, or cofilin which sequesters ADP-G-actin to prevent polymerisation (Didry *et al.*, 1998). A class of protein termed actin cappers, such a gelsolin, prevents further polymerisation by binding tightly to plus ends (Laine *et al.*, 1998). Proteins known as actin cross-linking and bundling proteins promote the formation of different higher-order actin assemblies associated with different cellular structures (**Figure 2**). These structures play fundamental roles in many cellular processes, including cell migration, cytokinesis, exocytosis, endocytosis and phagocytosis.

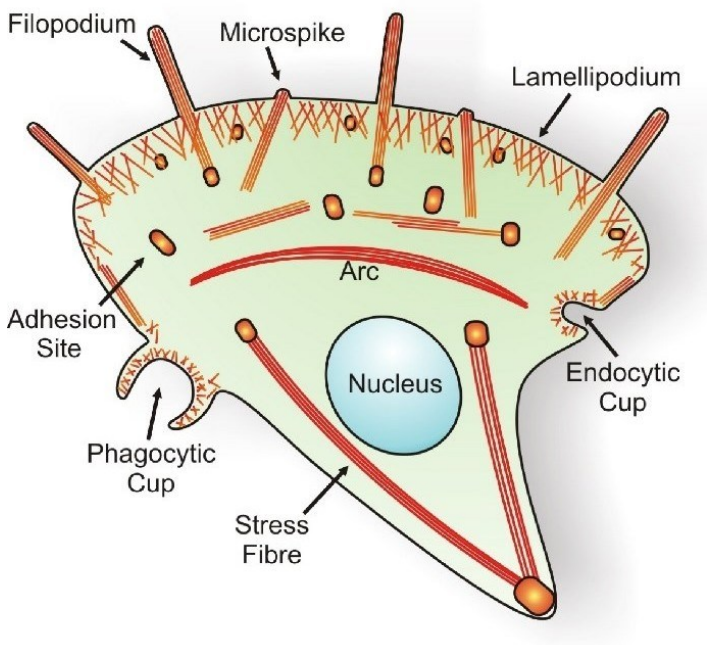

Figure 2. Organization of actin-enriched cellular structures. Schematic depicting distinct F-actin-containing cellular structures. Adapted from (Faix *et al.*, 2009)

At the leading edge of the cell the "lamellipodium" forms a large sheet-like membrane protrusion formed by a dense network of branched actin polymerising towards the membrane to drive cell movement forward (Innocenti, 2018). The lamellipodium is commonly associated

with “microspikes”, structures that span the lamellipodium without protruding beyond the cell edge and comprising parallel actin bundles (Small *et al.*, 2002). Microspikes can convert into “filopodia”, elongated parallel actin bundles forming thin finger-like membrane protrusions involved in probing the microenvironment (Blake and Gallop, 2023).

Other important actin structures are the ventral stress fibers, a contractile network of actin bundles cross-linked by α-actinin and the motor protein myosin II, which connect the cell cytoskeleton to the extracellular matrix (ECM) via focal adhesion (FA) sites (Michelot and Drubin, 2011). Transverse arcs, another subtype of stress fibers, are curved bundles of actin and myosin filaments driving retrograde actin flow in actively protruding cells (Michelot and Drubin, 2011).

The cell cortex, a dense layer of branched actin network under the cell membrane, is important for the maintenance and generation of cell shape change during processes such as endocytosis (Mylvaganam *et al.*, 2021). Phagocytosis is a form of endocytosis where large particles are engulfed (such as cell debris or bacteria). The interaction of particles with cellular surface receptors triggers actin polymerisation which generate pseudopodia, structures enriched in cross-linked actin that will ultimately surround the adhered particle to form a phagocytic cup (Barger *et al.*, 2020).

In contrast to phagocytosis, another form of endocytosis termed receptor-mediated endocytosis is involved in the selective uptake of fluids or macromolecules in smaller vesicles. The best characterised process is clathrin-mediated endocytosis, where several adaptors and scaffold proteins assemble, leading to the formation of a clathrin lattice that curves the plasma membrane and drives membrane invagination, forming an endocytic pit (Kaksonen and Roux, 2018). Studies have demonstrated that actin regulates multiple stages of endocytosis, including

invagination, elongation of the endocytic vesicle and closure (Collins *et al.*, 2011; Yoshida *et al.*, 2018; Yang *et al.*, 2022).

Overall the interplay of different actin-associated proteins promotes the formation of different actin structures within the cell which are essential for key processes including cell shape maintenance, cell adhesion and migration.

1.1.2. Microtubules

Microtubules, the stiffest cytoskeleton component with a diameter of about 25 nm, are hollow cylindrical structures composed of heterodimers of α- and β-tubulin that are arranged head-to-tail into protofilaments (Brouhard and Rice, 2014) (**Figure 1B**).

The microtubule network is mostly known for its role in the separation of chromosomes during mitosis and the intracellular transport of organelles. In this case, microtubule associated proteins (MAPs) such as kinesin-1 and dynein are molecular motors fuelled by the energy of ATP hydrolysis, to carry cargoes toward microtubule plus ends and minus ends respectively (Gennerich and Vale, 2009).

Microtubule minus ends are associated to microtubule-organising centres near the cell centre, while the highly dynamic plus ends extend towards the cell periphery, with repeated phases of growth and shrinkage in a process known as dynamic instability (Mitchison and Kirschner, 1984). This process is driven by the hydrolysis of guanosine triphosphate (GTP) associated to tubulin (Hyman et al., 1992). Regulation of microtubule polymerisation and stability involves addition of post-translational modifications, activity of MAPs, as well as microtubule plus-end-tracking proteins (+TIPs) which interact with the extremities of microtubules to regulate their growth (Ramkumar *et al.*, 2018).

1.1.3. Intermediate filaments

Intermediate filaments (**Figure 1C**) are a diverse family of proteins which include keratins, vimentin, desmin, lamin, and neurofilaments. There are more than 70 genes coding for intermediate filament proteins, which are categorized into six subgroups (Type I to VI) based on sequence homology (Herrmann *et al.*, 2009).

Unlike F-actin filaments and microtubules that are made of globular proteins, intermediate filaments are assembled from elongated coiled-coils fibrous proteins, where tetramers assemble laterally into the unit-length filament (ULF), which then associate to form non-polar filaments with an average diameter of ~10 nm. Intermediate filaments are linked to other components of the cytoskeleton through anchor proteins called plectin and they associate to the outer nuclear membrane via nesprin-3 (Morgan *et al.*, 2011; Wiche, 2021).

Intermediate filaments are non-polarized structures and usually not dynamic, forming a stable network throughout the cytoplasm. The main function of intermediate filaments is to protect cells from mechanical stress, thus providing structural support and stability to cells.

1.1.4. Septins

Septins, viewed as a fourth component of the cytoskeleton, are a group of GTP-binding proteins which polymerise to form typically 4nm thick non-polar filaments (**Figure 1D**). They can also assemble in more complex structures such as paired filaments, rings and gauzes and play key roles in numerous biological processes such as mitochondrial fission, cell division and motility (Mostowy and Cossart, 2012; Spiliotis and Nakos, 2021). They are also involvedin various human diseases including neurodegenerative disorders, infertility and cancer (Angelis and Spiliotis, 2016).

Septins, which act as scaffold for protein recruitment and as a diffusion barrier for subcellular compartmentalization, interact with different actomyosin structures including the contractile ring, cortical arcs, phagocytic cups and actin stress fibers (Joo *et al.*, 2007; Huang *et al.*, 2008; Dolat *et al.*, 2014). *In vitro*, studies have shown that septins bind to membranes presenting micron-scale curvature, highlighting an intrinsic curvature-sensing ability of septins (Bridges *et al.*, 2016; Lobato-Márquez *et al.*, 2021). This preference for micrometer-scale curvature is attributed to an amphipathic helix domain in the C-terminal extension of SEPT6 in humans and its homologue Cdc12 in yeast (Bridges et al., 2016; Lobato-Márquez et al., 2021).

Humans have 13 functional genes (*Sept1-Sept12* and *Sept14*) encoding septin proteins (30–65 kDa), which can be classified into four groups based on sequence similarity: SEPT2 group (SEPT1, SEPT2, SEPT4, SEPT5), SEPT3 group (SEPT3, SEPT9, SEPT12), SEPT6 group (SEPT6, SEPT8, SEPT10, SEPT11, SEPT14) and SEPT7 group (which contains only one member, SEPT7). Septin subunits from the different subgroups interact through their G (containing the GTP-binding domain) and NC (containing the amino- and carboxy-terminal regions) interfaces to form rod-shaped, palindromic hetero-hexamers or hetero-octamers (Soroor *et al.*, 2021). The hexamer is composed of SEPT2-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2 and the octamer is SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-SEPT2. On the C-terminus, there is a domain of unknown function, the septin unique element (SUE), and the N-terminus contains the polybasic region (PBR), a proline-rich domain. Due to alternative translation start and splice sites, septin isoforms can be generated (in the case of SEPT9, five isoforms are known, SEPT9_i1 to _i5), characterised by the length and sequence of their N terminus and by their distinct functions (McIlhatton *et al.*, 2001; Verdier-Pinard *et al.*, 2017).

In human, native septins isolated from cells exist in the form of stable hexamers and octamers (Sellin *et al.*, 2011; Sellin *et al.*, 2014). In yeast, using fluorescence spectroscopy and total internal reflection fluorescence microscopy, current model suggests that cytosolic septins are

also likely complexes (not monomers or filaments). On the plasma membrane, short septin filaments merge into longer filaments and other higher-order assemblies (Bridges *et al.*, 2014).

How septins are regulated is still poorly understood. Studies have shown that septin functions can be regulated by post-translational modifications such as phosphorylation or sumoylation which is important for filament bundling and cytokinesis (Ribet *et al.*, 2017; Sharma and Menon, 2023). Moreover, the dynamics of septin filaments and their interactions with stress fibers are regulated by the Rho-GTPase cell division cycle protein 42 (Cdc42) and its effectors Binder of Rho GTPases 2 (BORG2) and 3 (BORG3) (Salameh *et al.*, 2021). Disruption of septins by RNA interference leads to the loss of stress fibers and alters the organization and dynamics of microtubules (Kinoshita *et al.*, 2002; Kremer *et al.*, 2005; Spiliotis and Nakos, 2021).

1.2. Septins in host-pathogens interactions

A role for septins in bacterial infection first emerged approximately 20 years ago from studying *Listeria monocytogenes* invasion (Pizarro-Cerdá et al., 2002). Since then, septins have been associated with a wide variety of bacterial pathogens such as *Shigella flexneri*, *Chlamydia trachomatis, Clostridium difficile, Salmonella Typhimurium, Pseudomonas aeruginosa* and enteropathogenic *Escherichia coli*. In the following section we revisit the history of septin biology in the context of bacterial infection and emphasize how the study of microbial pathogenesis will continue to unveil key insights into septin biology.

1.2.1. The history of septin biology and bacterial infection

The content of this sub-chapter is published :

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The History of Septin Biology and Bacterial Infection

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Abstract

Investigation of cytoskeleton during bacterial infection has significantly contributed to both cell and infection biology. Bacterial pathogens *Listeria monocytogenes* and *Shigella flexneri* are widely recognised as paradigms for investigation of the cytoskeleton during bacterial entry, actin-based motility, and cell-autonomous immunity. At the turn of the century, septins were a poorly understood component of the cytoskeleton mostly studied in the context of yeast cell division and human cancer. In 2002, a screen performed in the laboratory of Pascale Cossart identified septin family member MSF (MLL septin-like fusion, now called SEPT9) associated with *L. monocytogenes* entry into human epithelial cells. These findings inspired the investigation of septins during *L. monocytogenes* and *S. flexneri* infection at the Institut Pasteur, illuminating important roles for septins in host–microbe interactions. In this review, we revisit the history of septin biology and bacterial infection, and discuss how the comparative study of *L. monocytogenes* and *S. flexneri* has been instrumental to understand septin roles in cellular homeostasis and host defense.

1. Introduction

1.1. Bacterial interactions with the actin cytoskeleton

Rearrangements of the host cell cytoskeleton during infection are well known to promote events that benefit a bacterial pathogen, including internalisation into the host cell and dissemination from cell-to-cell (Haglund & Welch, 2011). Over the past ~30 years, *Listeria monocytogenes* has emerged as a model bacterial pathogen used to study cytoskeleton rearrangements during infection, providing valuable insights into fundamental aspects of host cell biology (Radoshevich & Cossart, 2018). For example, *L. monocytogenes* has been critical

to reveal a role for clathrin in bacterial invasion (Veiga & Cossart, 2005), and a role for the actin-related protein 2/3 (ARP2/3) complex in actin-based motility (Welch, Iwamatsu, & Mitchison, 1997).

Invasive bacteria promote their internalisation into normally nonphagocytic host cells using two different mechanisms referred to as "zippering" and "triggering" (Cossart & Sansonetti, 2004). Zippering bacteria (such as *L. monocytogenes*) present invasion molecules that mimic endogenous ligands to host cell surface receptors, initiating signalling cascades that result in actin polymerisation and membrane extensions, which zipper around invading bacteria. Triggering bacteria (such as *Shigella flexneri*) rely on their type III secretion system (T3SS) to deliver effector proteins into the host cell cytosol that trigger actin polymerisation and membrane ruffling, engulfing invading bacteria in a process similar to macropinocytosis.

Following invasion, bacterial pathogens are enclosed within an internalisation vacuole (called the phagosome). In some cases, bacteria such as *L. monocytogenes* and *S. flexneri* escape to the host cell cytosol where they replicate and initiate actin-based motility by polymerisation ofactin at one pole of the bacterium (Welch & Way, 2013). In this way, actin-based motility enables cytosolic bacterial pathogens to escape cell-autonomous immunity and disseminate from cell-to-cell.

1.2 The septin cytoskeleton

As compared with actin, relatively little was known about the role of septins during bacterial infection. Septins were discovered by Nobel Laureate Leeland Hartwell while screening for cell division cycle (cdc) mutants in the budding yeast *Saccharomyces cerevisiae* (Hartwell, 1971). Septins have since been identified in all eukaryotes (except higher plants) and are considered a component of the cytoskeleton interacting with actin, microtubules, and cellular

membrane (Mostowy & Cossart, 2012). In humans, septins are classified into four groups (namely the SEPT2, SEPT3, SEPT6, and SEPT7 groups) based on sequence homology. Septin subunits from different groups interact through their G (consisting of the GTP-binding domain) and NC (consisting of the amino and carboxy-terminal regions) interfaces, forming non-polar complexes and filaments. Septin filaments can bundle and by interacting with cellular membrane form higher order structures, such as rings.

Investigations on septin molecular functions have highlighted their roles in numerous biological processes, including cell division and motility, by acting as scaffolds for protein recruitment and diffusion barriers for subcellular compartmentalization (Bridges & Gladfelter, 2015). Recent studies have illuminated septin roles in the organisation of actin and microtubule networks (Spiliotis, 2018). In agreement with septins being key for cellular homeostasis, septin dysfunction has been implicated in a wide variety of pathological conditions including cancer, male infertility, and neurodegenerative disorders (Dolat, Hu, & Spiliotis, 2014). A role for septins in bacterial infection was first suggested from studying *L. monocytogenes* invasion approximately 20 years ago in the laboratory of Pascale Cossart (Pizarro-Cerdá et al., 2002). Since then, septins have been associated with a wide variety of bacterial pathogens, including *Chlamydia trachomatis, Clostridium difficile, Salmonella Typhimurium* and enteropathogenic *Escherichia coli (*Boddy et al., 2018; Lee et al., 2017; Nölke et al., 2016; Volceanov et al., 2014) and play important roles in host cell infection (Torraca & Mostowy, 2016; Van Ngo & Mostowy, 2019; Figure 1). In this review, we discuss how the study of *L. monocytogenes* and *S. flexneri* at the Institut Pasteur launched septins into the field of bacterial infection.

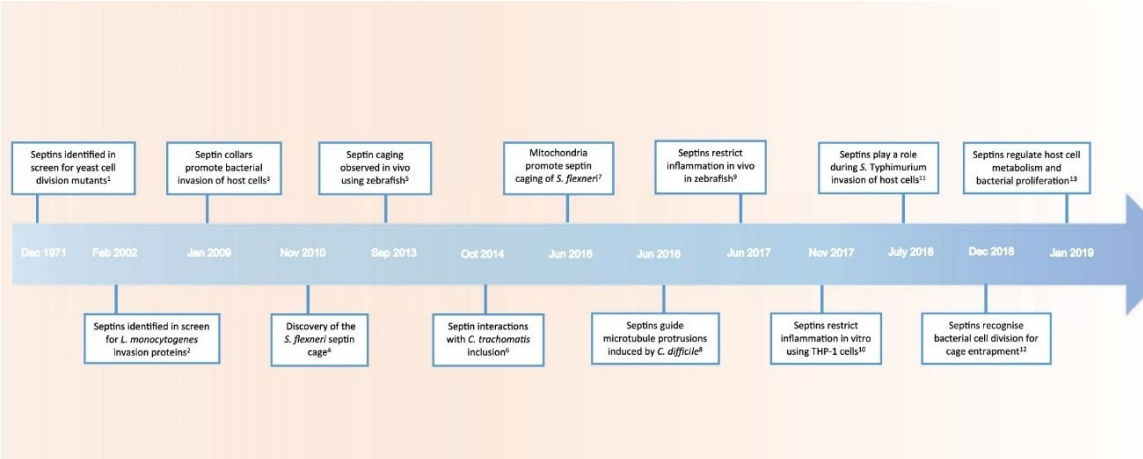

Figure 1. Timeline: A history of septin biology and bacterial infection. Septins were discovered using mutagenesis screens in yeast to find genes crucial for cell division (Hartwell, 1971). In the case of infection biology, septins were discovered at the Institut Pasteur using a proteomic screen to find genes crucial for *Listeria monocytogenes* entry (Pizarro-Cerdá et al., 2002). Septins assemble as collar-like structures around invading bacteria, launching septins into the field of bacterial infection (Mostowy, Tham, et al., 2009). Septins assemble into cage-like structures around actin-polymerising *Shigella flexneri*, revealing a new link between the cytoskeleton and cell-autonomous immunity (Mostowy et al., 2010). Septin caging observed *in vivo* using *S. flexneri* infection of zebrafish, a first animal model testing the role of bacterial autophagy *in vivo* (Mostowy et al., 2013). Septins organise filamentous actin around the *Chlamydia trachomatis* inclusion and are required for inclusion release by extrusion (Volceanov et al., 2014). Mitochondria promote septin assembly into cages that entrap *S. flexneri* for targeting to autophagy, a first report to highlight septin-mitochondria interactions (Sirianniet al., 2016). Septins guide microtubule protrusions induced by *Clostridium difficile* during host cell adhesion (Nölke et al., 2016). Septins discovered to restrict inflammation *in vivo* and protect zebrafishlarvae from *S. flexneri* infection (Mazon-Moya et al., 2017). Septin-mediated autophagy in THP-1 cellsobserved to restrict inflammation *in vitro* (Lee et al., 2017). Septins regulate actin dynamics to promote*Salmonella Typhimurium* entry in host cells (Boddy et al., 2018). Septins recognise micron-scale membrane curvature of dividing *S. flexneri* for delivery to lysosome, highlighting a new link between cell division and cell-autonomous immunity (Krokowski et al., 2018). Septins and the autophagy machinery can promote the proliferation of intracellular *S. flexneri* not entrapped in septin cages (Lobato-Márquez, Krokowski, Sirianni, Larrouy-Maumus, & Mostowy, 2019)

2. Septins and bacterial entry

2.1. Actin rearrangements during bacterial entry

How *L. monocytogenes* can enter nonphagocytic cells has been investigated in great detail (Cossart & Sansonetti, 2004; Radoshevich & Cossart, 2018). Several surface proteins contribute to this process, including internalin (InlA), which promotes bacterial entry through interaction with human E-cadherin, a transmembrane glycoprotein located at adherens

junctions and interacting with actin through catenins (Bonazzi, Lecuit, & Cossart, 2009; Gaillard, Berche, Frehel, Gouin, & Cossart, 1991). A second *L. monocytogenes* invasion protein is internalin B (InlB), which interacts with hepatocyte growth factor receptor (HGF-R / Met), a transmembrane receptor tyrosine kinase (Shen, Naujokas, Park, & Ireton, 2000). Both the InlA- and InlB-mediated entry pathways require activation of the ARP2/3 complex, inducing localised polymerisation of actin to promote bacterial engulfment.

2.2. Septin roles in bacterial entry: From recruitment to function

To identify new players in *L. monocytogenes* entry, the laboratory of Pascale Cossart used a proteomic approach based on isolation of internalisation vacuoles containing InlA- or InlB-coated latex beads (Pizarro-Cerdá et al., 2002). From this, it was discovered that septin family member MSF (MLL septin-like fusion), now referred to as SEPT9 (Macara et al., 2002), is enriched at the site of InlB-mediated entry. Subsequent experiments using fluorescent microscopy showed that SEPT9 is recruited to the entry site of InlB-coated beads, where it colocalises with actin. These observations suggested that SEPT9 regulates the InlB-mediated entry of *L. monocytogenes*. Consistent with this, studies using bacterial cells revealed that SEPT9, and its interacting partners SEPT2 and SEPT11, are recruited as 0.6 μm collar-like structures with actin at the entry site of *L. monocytogenes* (Mostowy, Tham, et al., 2009; Figure 2 [L1]). Septin collars were similarly detected at the entry site of triggering bacteria, including *S. flexneri* (Figure 2 [S1]). Considering that septins function as heterooligomers and the position of SEPT9 in the human septin complex was not yet clear, studies initially focused on SEPT2 because it was viewed as central for septin complex formation and function in human cells (Sirajuddin et al., 2007). The depletion of SEPT2 using small interfering RNA (siRNA) significantly decreased the entry of *L. monocytogenes* and *S. flexneri*, revealing that SEPT2

can promote bacterial entry (Mostowy, Tham, et al., 2009). Surprisingly, a separate study showed that SEPT11 restricts InlB-mediated entry by *L. monocytogenes*, highlighting that different septins may have different roles during bacterial entry (Mostowy, Danckaert, et al., 2009).

Importantly, treatment of cells with cytochalasin D (an inhibitor of actin polymerisation) impaired septin recruitment to the site of bacterial entry, indicating that actin polymerisation precedes septin assembly. Using fluorescence resonance energy transfer (FRET), it was shown that SEPT2 can activate PI 3-kinase signalling during InlB-Met interactions (Mostowy, Danckaert, et al., 2009). Using atomic force microscopy (AFM), it was shown that septins anchor Met to the cortical actin cytoskeleton, where it may regulate surface receptor dynamics (Mostowy et al., 2011). At the plasma membrane, septins may act as a diffusion barrier to restrict the localization of phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2)-enriched microdomains crucial for signalling processes (Sharma et al., 2013). Recent work using *S. Typhimurium* showed that septins are involved in membrane ruffle morphology and signalling through the actin nucleating protein FHOD1 (Boddy et al., 2018). Taken together, the septin cytoskeleton can be viewed as a molecular platform modulating cytoskeletal dynamics and signal transduction events during bacterial entry. These conclusions are in agreement with work showing that septins assemble into collar-like structures at the base of the phagocytic cup during FcγR-mediated phagocytosis in macrophages and neutrophils (Huang et al., 2008).

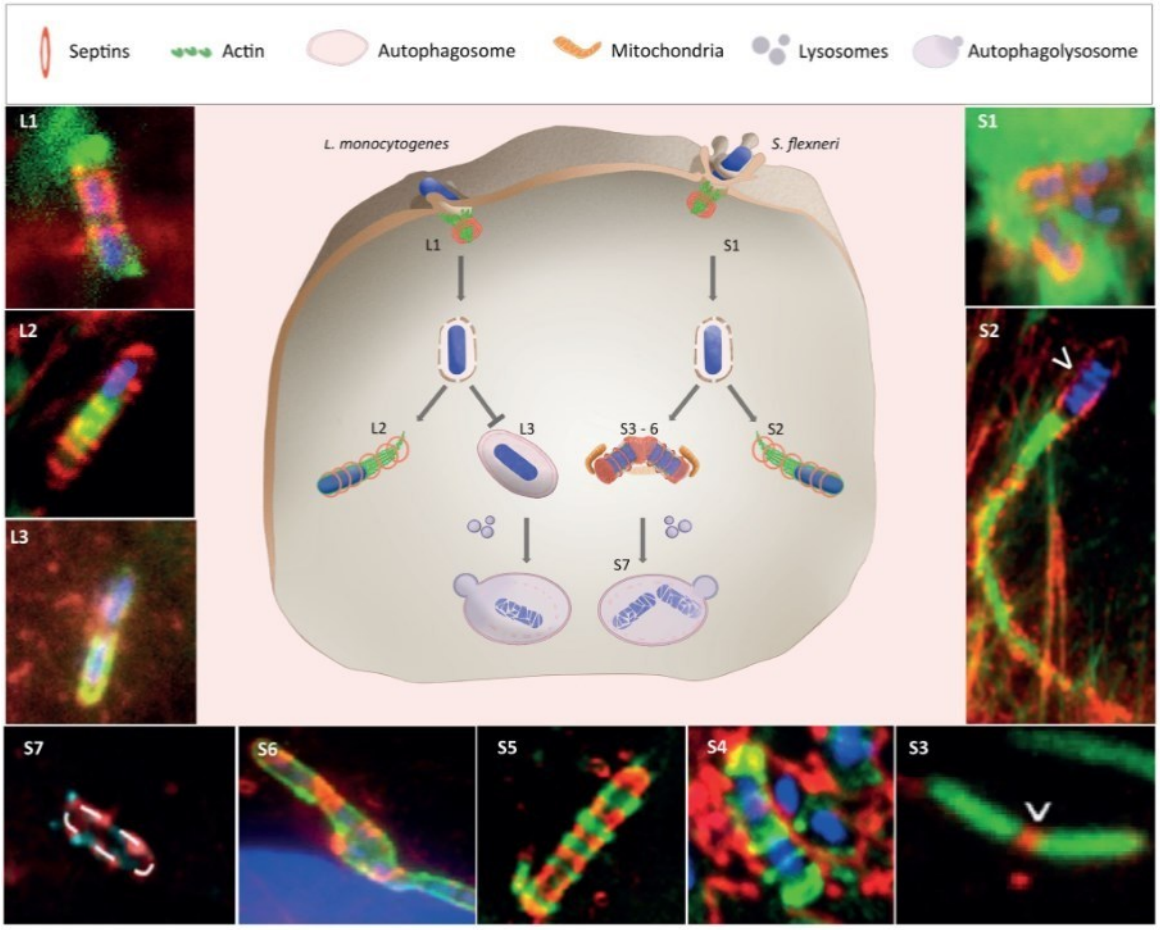

Figure 2. *Listeria monocytogenes* and *Shigella flexneri* interactions with the septin cytoskeleton. (Centre) Model illustrating the interaction of *L. monocytogenes* (left) and *S. flexneri* (right) with septins during infection of host cells. (Surrounding) Representative immunofluorescence micrographs of septin-bacteria interactions. (L1, S1) Septin recruitment to the site of *L. monocytogenes* and *S. flexneri* entry in Jeg-3 and HeLa cells, respectively. Endogenous septin stained with anti-SEPT9 (L. monocytogenes) or anti-SEPT2 (*S. flexneri*), actin stained with anti-F-actin (green), and bacteria marked using DAPI (blue). Images adapted with permission from (Mostowy, Tham, et al., 2009). (L2,S2) Septin recruitment to actin tail of *L. monocytogenes* and *S. flexneri* in Jeg3 cells and HeLa cells, respectively. Endogenous septin stained with anti-SEPT9, actin stained with phalloidin (green), and bacteria marked using DAPI (blue). Images adapted with permission from (Mostowy et al., 2010). (L3)Recruitment of autophagy markers to *L. monocytogenes* ΔactA in HeLa cells. p62 stained with anti-p62(green), NDP52 stained with anti-NDP52 (red), and bacteria marked using DAPI (blue). Image adaptedwith permission from (Mostowy et al., 2011). (S3) Septins are recruited to micron-scale curvature of dividing intracellular bacteria. SEPT6-GFP HeLa cells were infected with *S. flexneri* mCherry. White arrowheads indicate septin recruitment to the bacterial division site. Image adapted with permission from (Krokowski et al., 2018). (S4) Mitochondria promote septin cage assembly. HeLa cells stably expressing SEPT6-GFP were transfected with Mito-BFP and infected with *S. flexneri* mCherry. SEPT6 is shown in green, mitochondria in red, and *S. flexneri* mCherry in blue. Image adapted with permission from (Sirianni et al., 2016). (S5) The *S. flexneri* septin cage. Endogenous septin stained with anti-SEPT9, actin stained with phalloidin (green), and *S. flexneri* marked using DAPI (blue). Image adapted with permission from Mostowy et al. (2010). (S6) Septin caged bacteria are targeted to autophagy in HeLa cells expressing GFP-LC3B. Endogenous septin stained with anti-SEPT2 (red) and *S. flexneri* marked using DAPI (blue). Image adapted with permission from (Mostowy et al., 2010). (S7) Septins target bacteria to lysosomal degradation. SEPT6-GFP HeLa cells labelled with LysoTracker red. Septin cage-entrapped *S. flexneri* is shown as dashed white outline. Image adapted with permission from Krokowski et al. (2018)

3. Septins and actin-based motility

3.1. The paradigm of the bacterial actin tail

The observation that septins form collar-like structures around invading bacteria initiated great interest in the role of septins during other actin-based strategies exploited by intracellular pathogens, such as actin-based motility. Intracellular bacterial movement was first observed in the 1950s in cells infected with *Rickettsia rickettsia*, the causative agent of Rocky Mountain spotted fever (Schaechter, Bozeman, & Smadel, 1957), and direct evidence for bacterial actin-based motility came in the 1980s from cells infected with *L. monocytogenes* (Tilney & Portnoy, 1989) and *S. flexneri* (Bernardini, Mounier, D'Hauteville, Coquis-Rondon, & Sansonetti, 1989).

In the case of *L. monocytogenes*, actin tail formation requires expression of the surface protein ActA at the bacterial cell pole, which acts as a mimic of the nucleation promoting factor Wiskott–Aldrich syndrome protein (WASP) to recruit and activate the ARP2/3 complex (Welch et al., 1997). In contrast, *S. flexneri* expresses the bacterial autotransporter protein IcsA (also called VirG), whose activity recruits the neural WASP (N-WASP) and consequently, ARP2/3 to the bacterial cell pole (Bernardini et al., 1989; Makino, Sasakawa, Kamata, Kurata, & Yoshikawa, 1986). Polar localisation of IcsA is essential for actin tail formation, and a recent study showed that polymerisation of MreB (the bacterial actin homologue) promotes polar IcsA positioning for actin tail formation (Krokowski et al., 2019).

Actin-based motility is used by various pathogens for dissemination within and between cells (Gouin, Welch, & Cossart, 2005; Welch & Way, 2013). However, infected host cells have powerful mechanisms to restrict actin-based motility, highlighting unforeseen aspects of cell-autonomous immunity.

3.2. Enter the *Shigella* septin cage

Consistent with studies showing that septins are recruited to actin rich sites of bacterial entry, septins form ring-like structures around approximately 25% of *L. monocytogenes* and *S. flexneri* actin tails (Mostowy et al., 2010; Figure 2 [L2 and S2]). The depletion of SEPT2 or SEPT9 had no significant impact on bacterial directionality or movement, indicating that septin recruitment is not essential for actin-based motility. In agreement with this, septins were not detected among proteins identified by mass spectrometry of *L. monocytogenes* actin tails (Van Troys et al., 2008). Importantly, *L. monocytogenes* Δ*actA* and *S. flexneri* Δ*icsA*, isogenic mutants unable to polymerise actin tails, both fail to recruit septin (Mostowy et al., 2010). Together, these experiments suggested that septin rings assemble at sites of actin polymerisation and depend on actin polymerisation.

Strikingly, cytosolic *S. flexneri* surrounded by an actin cloud, though without an actin tail, are surrounded by septin cage-like structures (Figure 2 [S5]). In this case, septin cage-like structures compartmentalise approximately 15–30% of cytosolic *S. flexneri* (Mostowy et al., 2010). SEPT2 or SEPT9 depletion significantly increased the number of *S. flexneri* with actin tails, indicating that septins restrict actin tail formation. Similarly, inhibiting myosin II function using siRNA or blebbistatin increased the number of *S. flexneri* with actin tails. Treatment of cells with tumour necrosis factor α (TNFα), a pleiotropic cytokine shown to stimulate association of myosin II to SEPT2, promotes septin caging and restricts actin tail formation (Mostowy et al., 2010). These results highlight septin caging as a host defense mechanism counteracting actin-based motility to restrict the dissemination of *S. flexneri*. Since its discovery in *S. flexneri*-infected tissue culture cells, septin cage-like structures have been observed *in vivo* in *S. flexneri*-infected zebrafish larvae (Mostowy et al., 2013) and have also been observed in tissue culture cells infected with *Mycobacterium marinum* (Mostowy et al.,

2010), enteropathogenic *E. coli* (Lee et al., 2017), and vaccinia virus (Pfanzelter, Mostowy, & Way, 2018). In the case of vaccinia virus, septins exert their antiviral effect by forming rings around virions at the plasma membrane to suppress their release from infected cells.

Discovery of the *S. flexneri* septin cage revealed a new role for the cytoskeleton in cell-autonomous immunity by inhibiting actin-based motility. Since then, another mechanism inhibiting actin-based motility has been described: the coating of cytosolic *S. flexneri* by the interferon-inducible GTPase family of guanylate-binding proteins (GBPs; Li et al., 2017; Wandel et al., 2017; Piro et al., 2017). In this case, *S. flexneri* antagonises GBP-mediated cellular defenses by secreting the E3 ubiquitin ligase IpaH9.8, which ubiquitinates and degrades GBPs in a proteasome-dependent manner. In the future, it will be of great interest to explore the relationship between septin cages and GBP coats.

Why do only a fraction of cytosolic *S. flexneri* recruit septins? Considering that bacterial pathogens often manipulate host cell proteins and processes to evade cell-autonomous immunity (Huang & Brumell, 2014), it is highly likely that *S. flexneri* has evolved mechanisms to evade septin cage entrapment. For example, post-translational modification of host cell proteins by bacterial pathogens is well known to interfere with protein activity (Salomon & Orth, 2013). Septins are regulated by a variety of post-translational modifications including acetylation, phosphorylation, ubiquitination, and sumoylation (Koch, Acebron, Herbst, Hatiboglu, & Niehrs, 2015). New work has shown that the *S. flexneri* effector IcsB is an 18-carbon fatty acyltransferase catalysing the lysine Nε-fatty acylation of SEPT7 and SEPT11 (Liu et al., 2018). Whether post-translational modifications may affect septin cage assembly, and whether bacteria can interfere with this process, remains to be determined.

4. Other septin roles in cell-autonomous immunity

4.1. Septins and autophagy

Autophagy delivers cytosolic material enclosed in a double-membraned vacuole (called the autophagosome) to the lysosome for degradation (Levine, Mizushima & Virgin, 2011). In this way, autophagy acts as a cytosolic quality control mechanism, eliminating protein aggregates, damaged organelles, and intracellular microbes to maintain cellular homeostasis. Targeting of bacteria to autophagy can proceed through ubiquitin-dependent and ubiquitin-independent signalling pathways (Khaminets, Behl, & Dikic, 2016). Ubiquitinated bacteria are recognised by autophagy receptors such as p62 (sequestosome 1 or SQSTM1) and NDP52 (nuclear dot protein, 52 kDa), pattern recognition receptors called sequestosome 1/p62-like receptors (SLRs) that recruit membranes for autophagosome formation through their interaction with ATG8 family proteins such as LC3B (Johansen & Lamark, 2019). Targeting of bacteria to autophagy can also proceed through ubiquitin-independent pathways, such as LC3-associated phagocytosis (LAP) or opsonization by complement (Mostowy, 2013; Sorbara et al., 2018).

However, many intracellular bacteria have mechanisms to avoid recognition by autophagy (Huang & Brumell, 2014). In the case of *L. monocytogenes*, avoidance of autophagy is mediated via ActA by promoting recruitment of the actin-polymerisation machinery on the bacterial surface and also by enabling bacteria to move away from autophagic membranes via actin-based motility (Cheng, Chen, Engström, Portnoy, & Mitchell, 2018; Yoshikawa et al., 2009; Figure 2 [L3]). In addition to ActA, *L. monocytogenes* phospholipase C can reduce the intracellular levels of phosphatidylinositol 3-phosphate (PtdIns3P), a lipid required for LC3B lipidation (Mitchell et al., 2015; Tattoli et al., 2013). In the case of *S. flexneri*, avoidance of autophagy is mediated via the bacterial effector protein IcsB, which prevents the recruitment of ATG5 (a protein critical for autophagosome maturation) to IcsA (Ogawa et al., 2005). A

more recent study discovered that Nε-fatty acyltransferase activity of IcsB modifies CHMP5 (a component of the ESCRT-III complex) to escape from autophagy (Liu et al., 2018). In addition to IcsB, *S. flexneri* VirA can inactivate Rab1 to counteract autophagy (Dong et al., 2012).

Several autophagy markers (e.g., p62, NDP52, and LC3B) are recruited to septin cages (Mostowy et al., 2010; Mostowy, Sancho-Shimizu, et al., 2011; Figure 2 [S6]), and *S. flexneri* IcsB mutants are compartmentalised in septin cages more efficiently than the wild-type strain (Mostowy et al., 2010), consistent with a role for IcsB in autophagy escape (Liu et al., 2018; Ogawa et al., 2005). Strikingly, when septins (SEPT2 or SEPT9) or autophagy components (p62, ATG5, ATG6, or ATG7) are depleted by siRNA, both septin cages and autophagy markers fail to recruit around *S. flexneri* (Mostowy et al., 2010). These results highlight an interdependence between septin assembly and autophagy. In agreement with this, a study using proteomics found autophagy and mitochondrial proteins associated with septins in *S. flexneri*-infected cells (Figure 2 [S4]), and that actin-polymerising *S. flexneri* fragment mitochondria to escape from septin cage-mediated autophagy (Sirianni et al., 2016).

In a first study to investigate bacterial autophagy *in vivo*, infection of zebrafish larvae with *S. flexneri* highlighted autophagy as a crucial component of innate immunity at the whole animal level (Mostowy et al., 2013). Here, depletion of p62 using morpholino oligonucleotide significantly reduced the number of septin cage-associated *S. flexneri* and significantly increased bacterial burden. In agreement with the view that septin cages are antibacterial, work using HeLa cells showed that approximately 50% of *S. flexneri* entrapped in septin cages are not metabolically active (Sirianni et al., 2016). On the other hand, septins and the autophagy machinery can regulate metabolic pathways that promote the proliferation of intracellular *S. flexneri* not entrapped in septin cages (Lobato-Márquez et al., 2019).

Although the fate of *S. flexneri* in septin cages is relatively well characterised, how septins sense cytosolic bacteria was mostly unknown. It has been recognised for approximately 10 years that septin assembly is membrane facilitated (Tanaka-Takiguchi, Kinoshita, & Takiguchi, 2009), and recent work suggested that septins sense micron-scale curvature at the plasma membrane (Bridges, Jentzsch, Oakes, Occhipinti, & Gladfelter, 2016). In agreement with this, a recent study showed that septins are recruited to regions of micron-scale curvature presented by dividing bacterial cells (Figure 2 [S3]), and cardiolipin (a curvature-specific phospholipid) promotes septin recruitment to these regions (Krokowski et al., 2018). Following pharmacological inhibition of bacterial cell division, septins are recruited to bacterial cell poles but fail to assemble into cages, indicating that bacterial cell growth is required for septin cage entrapment. Therefore, bacterial cell division can be viewed as a fundamental danger signal used by the host cell to recognise intracellular bacteria (Krokowski et al., 2018; Krokowski & Mostowy, 2019; Van Ngo & Mostowy, 2019).

4.2. Septins and inflammation

A growing number of studies have indicated that the cytoskeleton plays key roles during inflammation (Mostowy & Shenoy, 2015). For example, actin has been linked to inflammation control through regulation of the NACHT, LRR, and PYD domain-containing protein 3 inflammasome (NLRP3), a large multimeric complex that activate caspase-1 and pro-inflammatory cytokines (Burger, Fickentscher, De Moerloose, & Brandt, 2016). Following cellular damage or infection, inflammasome formation and pyroptosis (an inflammatory type of programmed cell death) is linked with disruption of actin and microtubules (Davis et al., 2019). Although a role for septins in inflammasome activity has not yet been shown, recent studies have linked septins to dysregulated inflammatory responses (Lee et al., 2017; Mazon-

Moya et al., 2017). In the case of human monocytic THP-1 cells infected with *S. flexneri*, disruption of WASP by short hairpin RNA (shRNA) reduced septin caging and increased inflammasome activity and host cell death (Lee et al., 2017). At the whole animal level, work using the *S. flexneri*-zebrafish infection model discovered an *in vivo* role for septins in inflammation control (Mazon-Moya et al., 2017). Here, depletion of Sept7b or Sept15 (zebrafish orthologues of human SEPT7) using morpholino oligonucleotide significantly increased host susceptibility to *S. flexneri* infection. Moreover, Sept15 morphants exhibit significantly increased caspase-1 activity and host cell death, indicating that septins restrict inflammation *in vivo*. Taken together, these results suggest that septins may interact with the inflammasome, and factors important in septin-mediated restriction of inflammation can represent novel drug targets for the control of bacterial infection and inflammatory disorders.

5. Conclusion and future perspectives

The study of infection by *L. monocytogenes* and *S. flexneri* has significantly advanced our understanding of cellular microbiology and cell-autonomous immunity. *In vitro* reconstitution of *L. monocytogenes* and *S. flexneri* actin-based motility has been essential to identify key proteins underlying actin polymerisation (Loisel, Boujemaa, Pantaloni, & Carlier, 1999;Welch, Rosenblatt, Skoble, Portnoy, & Mitchison, 1998). Similarly, *in vitro* reconstitution of the *S. flexneri* septin cage may yield important insights on the biochemical, molecular, and structural aspects of septin-microbe interactions. To elucidate the precise molecular basis of *S.flexneri* septin caging, the use of high resolution (such as cryo-electron tomography) and highcontent (to screen host and/or bacterial factors) microscopy techniques will be of great interest.Although this review is focused on *L. monocytogenes* and *S. flexneri* infection, it is essential to

understand the full breadth of microbial pathogens regulated by septin biology, and to test how widespread is the role of cell shape and membrane curvature in pathogen sensing (Torraca & Mostowy, 2016; Van Ngo & Mostowy, 2019). Our current knowledge of septin function during infection mostly derives from work performed *in vitro* using nonphagocytic cells (e.g., HeLa); future work should address septin function in immune cells (e.g., macrophages and neutrophils) and whole animal models (e.g., zebrafish and mice). Overall, the study of septin biology and bacterial infection has provided unexpected insights into septin biology, and septin recruitment by a wide range of pathogens will likely continue to highlight novel septin functions. This information may help to elucidate therapeutic treatments for infection, inflammation, and other human diseases in which septins have been implicated.

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Conflict of interest

The authors declare no conflict of interest.

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1.2.2. Conclusion

Investigating the cell biology of bacterial infection has significantly enriched our understanding of septin biology. The role of septins in bacterial infection primarily comes from the study of the Gram-negative bacterial pathogen *S. flexneri*, a paradigm for cellular microbiology studies. Despite intense investigation, several exciting questions remain. For example, given the curvature sensing ability of septins it would be important to investigate their involvement in curvature-dependent phagocytic events during invasion by various microbes. Moreover, purified septins have been shown to interact directly with *S. flexneri* where surface lipopolysaccharide (LPS) can restrict septin binding (Lobato-Márquez et al., 2021). Could septins also interact with Gram-positive bacteria considering their fundamental surface differences with Gram-negative bacteria? What are the range of bacterial surface components that control septin recruitment?

A recent study from our lab using HeLa cells overexpressing RFP-SEPT6 suggested that septins are recruited to micron scale curvature presented by intracellular *S. aureus* (Krokowski et al., 2018). However, no further investigations have been done beyond that preliminary observation and thus the role of septins in the context of *S. aureus* infection was mostly unknown. A better understanding of septin-*S. aureus* interactions is important since it has the potential to advance both our understanding of septin biology and development of therapeutic strategies against *S. aureus* infections.

In the following section we describe the multidrug-resistant pathogen *S. aureus*, a leading bacterial cause of death globally- a distinction that sets it apart from most other pathogenic microbes (Ikuta et al., 2022). We highlight its unique attributes making it an interesting model for uncovering novel aspects of septin biology.

1.3. *Staphylococcus aureus* host-pathogen interactions

1.3.1. *S. aureus* is an important human pathogen

From shaping discovery of the very first antibiotic (penicillin in 1928), to making its mark across a range of media including TV shows, *S. aureus* has emerged as a recognizable presence in popular culture raising public awareness about the critical issue of antibiotic resistance. Despite implementation of infection control policies over the past decades to curb the growing impact of *S. aureus*, the lack of approved therapies combined with reports of isolates displaying resistance to almost all approved antimicrobials has solidified its reputation as a major global public health threat.

S. aureus is a Gram-positive coccoid bacterium of the Firmicutes phylum, with a diameter that ranges between 0.5 – 1.5 μm and a propensity to grow in grape-like clusters from which the name *Staphylococcus* is derived (**Figure 3**).

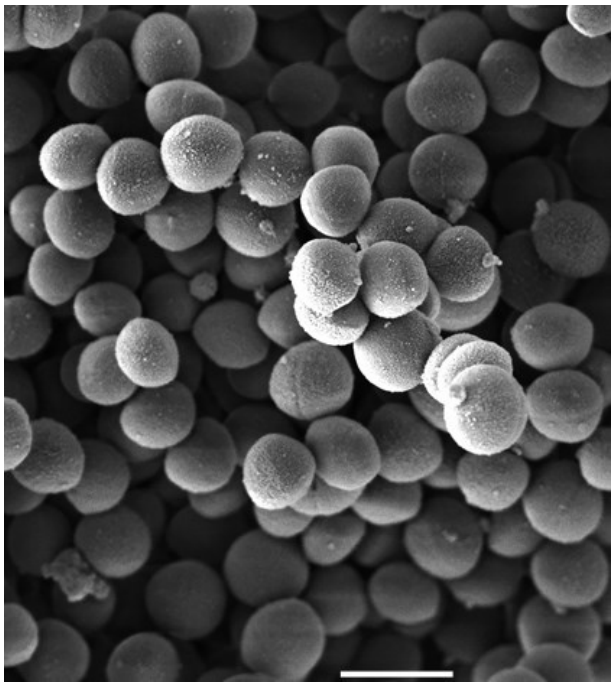

Figure 3. Scanning electron microscopy image of *Staphylococcus aureus*. Adapted from Norbert Bannert, Kazimierz Madela (Robert Koch Institute). Scale bar = 2 μm.

There are currently 85 recognised species and 28 subspecies of the genus *Staphylococcus*, but *S. aureus* remains the most frequently associated with human infection (Parte *et al.*, 2020). *S. aureus* is an important opportunistic pathogen and a common human commensal, colonizing approximately 20–40% of humans in the anterior nares (Wertheim *et al.*, 2005). *S. aureus* exhibits two distinct modes of growth: planktonic which is linked to acute infections, and biofilm which is associated with chronic infections (Archer *et al.*, 2011). In some cases, upon breach of the skin or in immunocompromised individuals, *S. aureus* can cause a wide range of diseases such as skin and soft tissue infections, pneumonia, osteomyelitis, bacteraemia and life-threatening infective endocarditis (Lowy, 1998). Notably, *S. aureus* bacteraemia led to a greater number of deaths than mortality from acquired immune deficiency syndrome (AIDS), tuberculosis, and viral hepatitis combined (van Hal *et al.*, 2012).

First identified in 1880 by Alexander Ogston in the pus of an acute abscess, *S. aureus* is currently recognised as a leading cause of bacterial infection both in community-acquired as well as hospital-acquired settings. This is due to the emergence of multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (Lee *et al.*, 2018). The World Health Organization (WHO) has listed MRSA and VRSA as "high priority" bacterial pathogens for which new therapeutic solutions are urgently needed (Tacconelli *et al.*, 2018).

To regulate its virulence and adapt to the local host microenvironment, *S. aureus* employs different regulatory systems such as the accessory gene regulator (Agr) quorum-sensing system (Howden *et al.*, 2023). *S. aureus* is equipped with an arsenal of virulence factors that include toxins, immune modulatory factors or adhesins that facilitate immune evasion and tissue adhesion during infection (Cheung *et al.*, 2021). The initial steps of adhesion and invasion of *S. aureus* into the host cell are pivotal for successful establishment of infection.

1.3.2. *S. aureus* invasion into the host cell

Although *S. aureus* has not been traditionally classified as an intracellular pathogen, many studies conducted over the past decades have highlighted that it can invade and persist in different cell types *in vitro* and *in vivo* (Hommes and Surewaard, 2022). These studies were limited to a small number of *S. aureus* strains and host cell types, using different infection protocols and timepoints. More recently, the intrinsic invasiveness of *S. aureus* was confirmed by a microscopy-based phenotypic profiling using a collection of clinical isolates. In this study, the majority of isolates (187 of the 191) were efficiently internalized by both non-professional and professional phagocytic cells, with a large proportion replicating and persisting within host cells (Rodrigues Lopes et al., 2022). In a mouse model, the ability to survive and replicate intracellularly has been shown to provide protection from antibiotics, promoting long-term colonization and *S. aureus* dissemination (Lehar *et al.*, 2015).

Intracellular *S. aureus* has also been associated with recurrent infections *in vivo* such as osteomyelitis and rhinosinusitis. Bacteria recovered from chronic infections often show heterogeneity in colony size, with a bacterial subpopulation that has previously been referred to as small colony variants (SCVs). SCVs originated by mutations in metabolic genes and adapted to persist in the intracellular milieu; they represent a quasi-dormant, slow-growing subpopulation characterised by high antibiotic tolerance and enhanced invasiveness due to overexpression of adhesins.

To invade cells, *S. aureus* possesses a diverse arsenal of adhesins, the most prevalent are cell wall (CW) anchored proteins belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) family. All MSCRAMMs share a similar structure mediating their attachment to components of the host ECM such as collagen, fibrinogen or fibronectin. The more clinically relevant are the well-documented fibronectin-binding proteins

A and B (FnBPA/B) which mediate invasion of a variety of non-professional phagocytic cells (NPPCs) such as epithelial, endothelial cells, fibroblasts, osteoblasts, and keratinocytes (Speziale and Pietrocola, 2020). Although the FnBPs-fibronectin-integrin α5β1 pathway is widely acknowledged to be the main invasion strategy, there are secondary mechanisms less studied involving the adhesins serine aspartate repeat-containing protein D (SdrD), clumping factor A (ClfA), serine-rich adhesin for platelets, Collagen adhesin A (Cna) (Josse *et al.*, 2017).

FnBPA/B has been shown to bind two predominant forms of fibronectin (Fn), cellular Fn (which is assembled into a fibrillar matrix) and the soluble plasma Fn (that is secreted and located in blood). The majority (~70%) of *S. aureus* clinical isolates express FnBPA/B which play crucial roles in surface adhesion *in vitro* and *in vivo*. In a mouse infection model, the ability of an FnBP mutant to colonise the kidneys and cause fatal sepsis is strongly impaired (Shinji *et al.*, 2011). In a rat model of valve endocarditis, expression of FnBPA is sufficient to confer virulence to the less pathogenic *Lactococcus lactis* (Que *et al.*, 2005). Additionally, it has been shown that integrin β1 accumulates on the surface of upper-airway epithelial cells from mice and humans with cystic fibrosis (CF), which may explain persistent *S. aureus* colonization in lungs of CF patients (Grassmé *et al.*, 2017).

One FnBP can potentially bind 11 Fn molecules, using them as a bridge to bind and cluster integrin α5β1 at the invasion site (Bingham *et al.*, 2008). It is not fully understood how downstream signalling is transduced, but evidence suggests that *S. aureus* co-opt FA signalling to drive cellular invasion (Murphy and Brinkworth, 2021).

C. *S. aureus* intracellular replication and survival

Although *S. aureus* can invade and persist in many cell types *in vitro* and *in vivo*, the life cycle of intracellular *S. aureus* is not fully understood and is highly dependent on the strain and

the infected host cell type (**Figure 4**). Professional phagocytes such as macrophage and neutrophils are a first line of host defense, delivering engulfed microbes into a phagosome to try and clear the infection. Following phagocytosis, *S. aureus* initially resides in early phagosomes presenting endosomal markers such as the small GTPase Rab5. This compartment will progressively mature into late phagosome and acquire markers such as lysosome-associated membrane protein 1 (LAMP1) and Rab7. Although some studies have shown an absence of acidification and recruitment of key lysosomal hydrolases, maturation into a phagolysosomal compartment has also been observed. *S. aureus* can withstand this microbicidal environment and replicate inside phagolysosomes by using various strategies involving generation of ammonia leading to deacidification, degradation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) during the oxidative burst. Additionally, *S. aureus* can modify its cell envelope properties to reduce interaction with antimicrobial peptides (AMPs). The survival of *S. aureus* within those "Trojan horse" phagocytes promotes dissemination and ultimately *S. aureus* kills the host cell from within and viable bacteria may start another cycle of intracellular infection.

In the case of NPPCs, intracellular survival of *S. aureus* has been shown in a wide variety of cell types, including epithelial cells, endothelial cells, osteoclasts, keratinocytes, and fibroblasts (Hommes and Surewaard, 2022). However, the intracellular fate of bacteria is different because cytotoxic *S. aureus* strains can escape the phagolysosomal compartment andreplicate in the cytosol (Strobel *et al.*, 2016). The quorum-sensing system Agr and its regulationof different toxins such as alpha-toxin and phenol-soluble modulins (PSMs) are required for escape into the cytosol (Grosz *et al.*, 2014). Additionally, a non-ribosomal peptide synthetase and its product phevaline were shown to be required for efficient phagosomal escape (Blattner*et al.*, 2016).

Cell type	Strain	Mechanism
Phagocytes		
BMDCs	PS80	Accumulation of autophagosomes permits intracellular survival and replication.
Murine alveolar macrophages MH-S	Newman	Delayed acidification of phagosomes and persistence inside acidic compartments.
THP-1	USA300 LAC	Survival within acidic phagosomes that increase *agr* expression and perturb phagolysosome formation.
RAW264.7	Mu3	Vancomycin resistance regulator VraR enhances the induction of autophagy for intracellular survival.
	USA300 LAC Newman	Growth observed in phagolysosomes dependent on acidic pH and the bacterial GraXRS regulatory system.
Human Monocyte derived Macrophages	Newman	Viable and dividing bacteria observed within vacuoles days after infection leading to host cell lysis.
Human PMN	USA300 LAC PS80	*S. aureus* resides in autophagosomes and delays apoptosis enabling prolonged intracellular survival.
Larval Zebrafish Neutrophils	SH1000	LC3-associated phagocytosis enables intracellular survival within non-acidified phagosomes.
Epithelial cells		
A459	Cowan	SCV and non-replicating persister formation in response to low pH of phagolysosomes enables persistence.
HaCaT	USA300 LAC	Survival observed in cytosol and autophagosomes. Selective advantage gained for *agr* mutants *via* autophagic suppression of the inflammasome.
HeLa	USA300 JE2	Intracellular survival, phagosomal escape and cytotoxicity dependent on multiple bacterial proteins.
293T	USA300 LAC 6850	Uses PSMα to escape host phagosome leading to bacterial replication in the host cytosol.
Endothelial cells		
EA.hy926	USA300 LAC	Replication within the host cytoplasm followed by cell lysis and escape. Non-dividing bacterial cells also observed intracellularly.
HUVECs	6850	SCVs fail to induce inflammatory response and persist intracellularly for several days.
Bone cells		
Murine Osteoclasts	USA300 LAC	Low intracellular bacterial number colocalized with phagolysosome whilst high bacterial number colocalized within non-acidic compartments.
Human osteocyte-like cells	WCH-SK2	Rapid generation of SCVs after intracellular invasion.

Figure 4. Intracellular survival of *S. aureus* in different professional and non-professsional phagocytic cells. Adapted from (Vozza *et al.*, 2021).

Cytosolic bacteria or damaged vacuolar membrane could be taken up by the autophagy machinery for degradation (Wang *et al.*, 2021). Autophagy is an evolutionarily-conserved eukaryotic homeostatic process in which damaged cellular organelles or unwanted cytosolic material (such as invading pathogens) are engulfed in double-membrane autophagosome and trafficked towards lysosomal degradation (Debnath *et al.*, 2023). In NPPCs ubiquitin associates with *S. aureus* which is then targeted by autophagy receptors, including sequestosome 1 (SQSTM1/p62), nuclear domain protein 52 (NDP52/CALCOCO2), and optineurin (OPTN) (Neumann *et al.*, 2016). However, to date there are conflicting results regarding the role of

autophagy in NPPCs infected with *S. aureus* which can be either beneficial or detrimental to the bacteria.

A more comprehensive understanding of mechanisms underlying both internalisation and intracellular survival is essential for advancing development of antimicrobial therapies against *S. aureus*. We propose that using *S. aureus* to study septin biology will provide new insights into septin functions during infection.

1.3. Aim of the study

Historically, investigation of the eukaryotic cytoskeleton during microbial infection has enabled key discoveries in both cell and infection biology (Cossart *et al.*, 1996; Robertin and Mostowy, 2020). Epithelial cells are viewed as the first physical barrier against infection. Recently it was observed that *S. aureus* could interact with septins during infection of the epithelial cell line HeLa (Krokowski *et al.*, 2018). However, the precise role of septins during *S. aureus* infection remained unknown. *S. aureus* is a multifaceted pathogen expressing an array of virulence factors to colonize the host and establish persistent infection. *S. aureus* can invade cells mainly via the FnBPA/B-Fn-integrin α5β1 pathway, a mechanism which is yet to be fully deciphered. Once internalized, *S. aureus* can have diverse intracellular fates depending on the host cell type, including residence in lysosomes, autophagosomes or cytosolic escape. Given that intracellular *S. aureus* is suggested to be a major reservoir for chronic infections, understanding the mechanisms of both internalisation and intracellular survival is essential for development of novel antimicrobial therapies against *S. aureus*. Therefore, the aim of this thesis is to investigate septin interactions with *S. aureus* during infection of the well-established epithelial cell line HeLa, an amenable model historically used to study the pathogenesis of most intracellular bacteria. The specific aims of this thesis are as follows:

(i) To assess the role of septins during *S. aureus* invasion of host cells;

(ii) To investigate septin interactions with intracellular *S. aureus*;

(iii) To exploit our cell-free *in vitro* reconstitution system based on purified recombinant septin proteins to study septin interactions with the *S. aureus* cell surface.

By pursuing these objectives, we aim to deepen our understanding of the interplay between septins and *S. aureus* during infection and ultimately contribute towards development of more effective antimicrobial therapies, such as host-directed approaches modulating septin biology to efficiently target invasive *S. aureus*.

Chapter 2. Materials and Methods

2.1. Bacterial strains

Table 1. List of bacterial strains used in this study.

Bacterial strains	Genotype	Source/Reference
	S. aureus	
SH1000-mCherry	Derivative of *S. aureus* NCTC-8325-4 with repaired rsbU, Chromosomal mCherry geh::PmaIMmCherry, erythromycin resistant	S. Foster lab
RN6390 wild-type (WT)	*S. aureus* NCTC8325 derivative, rbsU, tcaR, cured of φ11, φ12, φ13	T. Palmer lab
JE2 WT	Plasmid-cured derivative of MRSA USA300 LAC	P. Pereira lab
JE2 Δ*tarO*	JE2 *tarO* null mutant; *tarO* encodes for a glycosyltransferase required for the first step of WTA biosynthesis	P. Pereira lab
JE2 Δ*ltaA*	JE2 *ltaA* null mutant; *ltaA* encodes for a flippase that mediates the translocation of the glycolipid that anchors LTA to the cell membrane	P. Pereira lab
JE2 Δ*srtA*	JE2 srt*A* null mutant; *srtA* encodes for a transpeptidase that covalently bind proteins containing a CW sorting signal to the CW	P. Pereira lab
JE2 Δ*capE*	JE2 capE null mutant; *capE* encodes for the enzyme catalyzing the first steps in the synthesis of the soluble capsule precursor UDP-L-FucNAc.	P. Pereira lab
JE2 Δ*mprF*	JE2 mprF null mutant; *mprF* encodes for the enzyme catalyzing synthesis of the cationic phospholipid LysPG and its translocation to the outer membrane leaflet.	P. Pereira lab
JE2 Δ*atl*	JE2 mprF null mutant*; atl* encodes for the major autolysin Major Atl, a PG hydrolase with amidase and glucosaminidase domains.	P. Pereira lab
JE2 Δ*pbp4*	JE2 pbp4 null mutant; *pbp4* encodes for a transpeptidase catalyzing peptide linkages between polymerized PG glycan chains	P. Pereira lab
JE2 Δ*oatA*	JE2 oatA null mutant; *oatA* encodes for enzyme catalyzing O-acetylation of PG	P. Pereira lab
	E. coli	
BL21 (DE3) pLys	F^- *ompT gal dcm lon hsdS*$_B$ ($r_B^- m_B^-$) pLysS, chloramphenicol resistant	ThermoScientific (#EC0114)

DH5α	F⁻ φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ⁻ *thi*-1 *gyrA96 relA1*	ThermoScientific (#18265017)
DH5α mCherry-Rab5	DH5α with plasmid encoding and constitutively expressing mCherry-Rab5, carbemicillin resistant	Addgene
DH5α YFP-CWT	DH5α with plasmid encoding and constitutively expressing YFP-CWT, ampicillin resistant	Addgene # 176689

2.2. Cell lines

Table 2. List of eukaryotic cell lines used in this study.

Cell line	Description	Source/Reference
HeLa	Human ATCC CCL-2	J. Mercer lab
HeLa GFP-SEPT6	Human, stable expression of GFP-SEPT6, puromycin resistance	M. Way lab
HeLa GFP-SEPT6 LifeAct-iRFP	Human, stable expression of GFP-SEPT6, LifeAct-iRFP, puromycin resistance	M. Way lab

2.3. Plasmids

Table 3. List of plasmids used in this study.

Plasmids	Description	Source/Reference
pnEA-vH_hSEPT2	Encodes for human SEPT2 with a N-terminal hexahistidine tag.	M. Mavrakis lab
pnCS-msGFP–SEPT6-SEPT7	Encodes for human SEPT6 with fused to a N-terminal monomeric superfolded green fluorescent protein (msGFP), and for human SEPT7 with a C-terminal streptavidin-tag II	M. Mavrakis lab
pGEX-4T-DuD4	Encodes for the DuD45 fragment of FnBPA with a N-terminal glutathione S-transferase (GST) tag	M. Aepfelbacher
mCherry Rab5	Encoding for human Rab5 fuse to a N-terminal mCherry	Addgene #49201

2.4. Antibodies

Table 4. List of antibodies used in this study.

Antibodies	Species	Application/Dilution	Source/Reference
Primary antibodies			
SEPT2	Rabbit	Immunofluorescence (IF); 1:200	P. Cossart lab
SEPT7	Rabbit	IF 1:100	IBL #18991
SEPT9	Rabbit	IF 1:200	P. Cossart lab
Phosphorylated Myosin light-chain II Ser 19	Mouse	IF 1:200	Cell Signaling #3675
Active Integrin α5β1 SNAKA51	Mouse	IF 1:500	Sigma-Aldrich MABT201
Integrin α5	Rabbit	IF 1:500, Western blot (WB) 1:1000	Abcam #EPR7854
Alexa Fluor 488 Anti-Integrin α5	Mouse	Flow cytometry 1:100	Invitrogen #53049642
Anti-Integrin β1	Mouse	WB 1:1000	
Alexa Fluor 647 Anti-Integrin β1	Mouse	Flow cytometry 1:100	Abcam #12G10
P62/SQSTM1	Mouse	IF; 1:200	BD Biosciences #610832
LAMP-1	Mouse	IF; 1:200	eBioscience™ #eBioH4A3)
Secondary antibodies			
Alexa-555-conjugated anti-rabbit antibody	Goat	IF; 1:300	Invitrogen A-11034
Alexa-488-conjugated anti-rabbit antibody	Goat	IF; 1:300	Invitrogen #10082602
Alexa-547-conjugated anti-rabbit antibody	Goat	IF; 1:300	Invitrogen #A27040
HRP-conjugated anti-mouse	Goat	WB 1:10 000	Dako #P0260
HRP-conjugated anti-mouse	Goat	WB 1:10 000	Dako # P0448

2.5. Transfection, molecular probes and drugs

Table 5. List of reagents used in this study.

Compound	Application	Concentration	Source
Oligofectamine	siRNA transfection reagent	3 µl in 200 µl reaction	Invitrogen #12252011
JET-Pei	DNA transfection reagent	4 µl in 300 µl reaction	Polyplus #101-10N
Alexa Fluor 488/555/647 Phalloidin	IF, staining of F-actin	3 U/ml	Fisher #A12379
Hoechst 33342	IF, staining of DNA	1 µg/ml	Thermo Scientific #62249
Cytochalasin D	IF, inhibitor of actin polymerisation	1 µg/ml	Invitrogen #PHZ1063
Blebbistatin	IF, Inhibitor of myosin IIA	50 µg/ml	Sigma B0560
UR214-9	IF, Inhibitor of septin polymerisation	50 µg/ml	R.K. Singh

2.6. Microbiological methods

2.6.1. Bacterial culture

Bacterial strains used in this study can be found in **Table 1**. All bacterial strains were stored at - 80°C in medium containing 25 % (v/v) glycerol. *S. aureus* strains were grown in trypticase soy (TCS) broth or on TCS agar plate at 37 °C, with shaking at 200 rotations per minutes (rpm) for culture in broth. *E. coli* strains were cultured in LB broth (Lennox, Invitrogen #12780052) and LB agar (1,5%) plates (Sambrook *et al.*, 1989). Bacteria were grown in broth at 37°C and 200 rpm. Where appropriate, medium was supplemented with antibiotics at the following concentrations: 100 µg/ml ampicillin, 50 µg/ml kanamycin, 30 µg/ml chloramphenicol, 50 µg/ml spectinomycin. Unless stated otherwise, 1 mM of Isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to exponentially growing *E. coli* (OD_{600} 0.4) induce gene expression.

2.6.2. Preparation and transformation of bacterial chemical-competent cells

E. coli were grown in LB until OD600 of 0.3 then the cultures were centrifuged at 4000 x g, room temperature (RT) for 15 min. After resuspending the pellet in 2.5 ml ice-cold 0.1 M $CaCl^2$, bacteria were incubated on ice for 1 h. Plasmid DNA was added to competent bacteria and samples were incubated on ice for 10 min. Bacteria were heat-shocked for 45 seconds at 42°C and incubated on ice for 2 min. Bacteria were recovered in 1 ml of SOC (Super Optimal broth with Catabolite repression, Invitrogen™ 15544034) medium for 1 h at 37°C. Samples were then plated on LB agar plates containing the appropriate antibiotic (to select transformed bacteria) and incubated overnight at 37°C.

2.7. Eukaryotic cells assays

2.7.1. Cell culture

Eukaryotic cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma D5796), supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific 10500064) in a humidified incubator equilibrated under standard conditions (5 % CO2 at 37 °Cat 37 °C). For HeLa cells stably producing GFP-SEPT6 and LifeAct-iRFP, medium was supplemented with 1 µg/ml puromycin.

2.7.2. siRNA transfection

For small interfering RNA (siRNA) treatment, the negative universal control (Fisher #10793457), SEPT2 (Fisher #AM16706-ID18228), SEPT7 (Fisher #10289574-ID10323) and

SEPT9 (Fisher #AM16706- ID18228)were used in this study. HeLa cells were seeded in 12-well plates at 4 x 10^4 cells per well in DMEM containing 10 % FBS and transfected with siRNA 18 h later. Oligofectamine (Thermo Fisher Scientific #REF) was used for siRNA transfections according to the manufacturer's instructions and HeLa cells were treated for 72h.

2.7.3. DNA transfection

HeLa cells were seeded in 12-well plates at 1 x 10^5 cells per well in a 12-well plates and transfected with plasmid DNA 24 h later. JetPei was used for DNA transfection (Polyplus #ref) according to the manufacturer's instructions and HeLa were treated for 24h.

2.7.4. Bacterial infection of HeLa cells

For fixed microscopy, HeLa cells (1 x 10^5) were seeded in 12-well plates (Thermo Scientific) containing 18 mm glass coverslips 18 h before the infection. Bacterial cultures were grown as described and cell cultures were infected with *S. aureus* at the indicated multiplicity of infection (MOI). Cells were centrifuged at 110 g for 5 min at RT and plates were placed at 37 °C and 5% CO2 for 15 min. Infected cultures were washed 2 times with phosphate-buffered saline (PBS, pH 7.4) and either processed for microscopy analysis or gentamicin/lysostaphin protection assay. For protection assays (**Figure 5**), lysostaphin (10 µg/ml) in DMEM + 10%fcs was added to washed cells for 10 minutes to lyse all extracellular or adherent *S. aureus.* After incubation cells were washed 3 times in PBS and incubated in DMEM + 10%FCS gentamicin (100 µg/ml)and were lysed in H20 with 0.1% Triton X-100. Cell lysates were serially diluted and plated on TCS agar plates and incubated overnight at 37°C to determine the number of CFU.

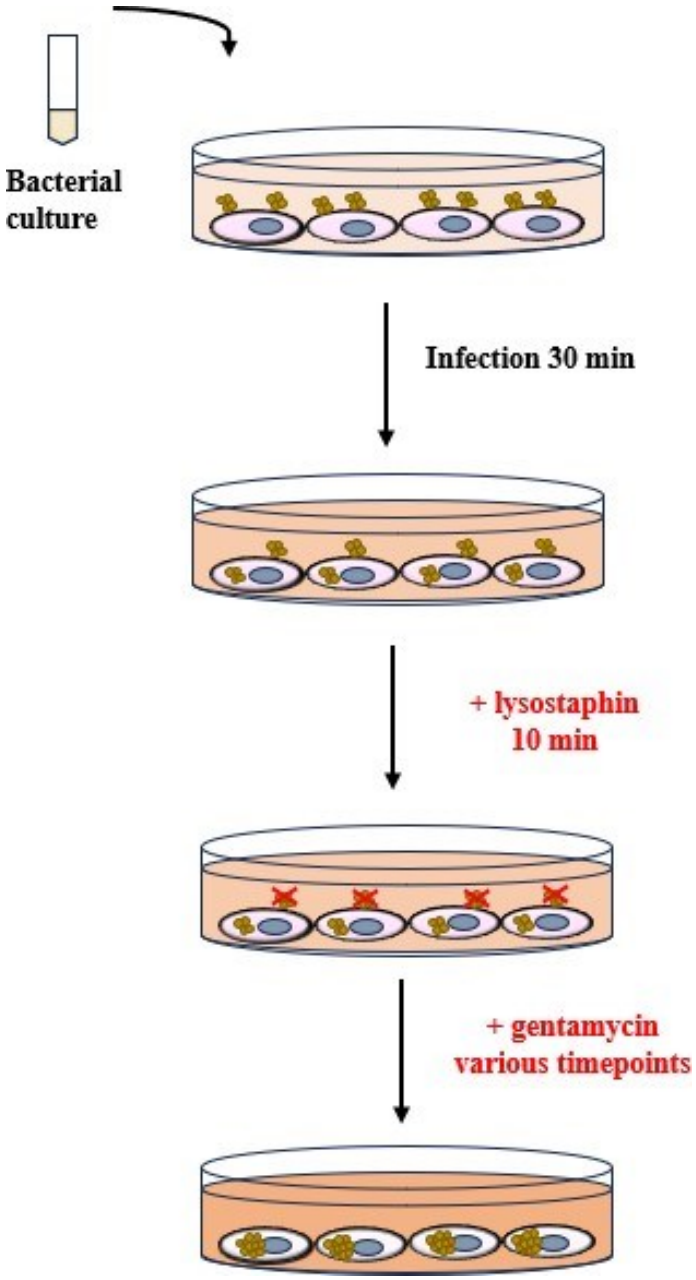

Figure 5. Diagram of the lysostaphin/gentamycin assay

2.7.4. Flow cytometry

Hela cells were treated with siRNA as described above for 72 h. Cells were harvested in a cooled centrifuge after trypsinisation and washed 3 times with cold washing buffer (1 x PBS

with 5% FBS). After washing, cells were resuspended in 100 µl of the ice-cold wash buffer (containing 0.125 µg of anti-integrin α5 antibody conjugated with Alexa Fluor 488 and 0.1 µg of anti-integrin β1 antibody conjugated with Alexa Fluor 647) and incubated at 4°C in the dark for 45 minutes. Cells were then washed 3 times with cold washing buffer before being analysed by flow cytometry with an LSRII (BD Biosciences) or Attune™ NxT (Thermo Fisher Scientific). The data were analysed using FlowJo software, version 10.7.1.

2.7.5. qRT-PCR

RNA was extracted with a RNeasy minikit (Qiagen) and reverse-transcribed using a QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Template cDNA was subjected to PCR using primers for gene encoding for integrin a5 (#HP205940, Origene), integrin b1 (#HP232479, Origene) and FAK (#HP208715, Origene). Quantitative reverse transcription PCR (RT-qPCR) was performed using 7500 Fast Real-Time PCR System machine and 7500 Fast Real-Time PCR software v2.3 (Applied Biosystems, Foster City, California) and SYBR green master mix (#10187094, Applied Biosystems). Primers against housekeeping gene GAPDH (Sirianni et al., 2016) and the delta-delta-Ct method were used for gene expression quantification (Livak and Schmittgen, 2001).

2.8. Immunofluorescence microscopy assay

Infected or uninfected cells were washed 3 times with PBS and fixed 15 min in 4% paraformaldehyde (in PBS) at RT. Fixed cells were washed 3 times with PBS and subsequently permeabilized 5 min with 0.1% Triton X-100 (in PBS). Cells were washed 3 times in PBS and then incubated for 1h in blocking buffer (PBS supplemented with 0.1% Triton X-100, 1% BSA

and 10% guinea pig serum). After blocking, cells were incubated 1 h 30 min with indicated primary antibody diluted in blocking buffer. Cells were then washed 9 times in PBS and incubated 1h with Alexa fluor conjugated secondary antibody diluted in blocking buffer. Cells were then washed 9 times in PBS and coverslips were mounted with ProLong Gold antifade reagent with DAPI (#P36935, Thermofisher). Fluorescence microscopy was performed using a 63x/1.4 C-Plan Apo oil immersion lens on a Zeiss LSM 880 confocal microscope driven by ZEN Black software (v2.3) or Axiovert Z1 driven by ZEN Blue 2.3 software (Carl Zeiss). Microscopy images were obtained using z-stack image series and confocal images were processed using Airyscan processing with "Auto Filter" and "3D Processing" options. Image analysis was performed using Fiji. For live microscopy, cells were grown on MatTek glass-bottom dishes (MatTek corporation). For imaging, medium was changed to FluoroBrite™ DMEM+10% FBS and acquisition was performed using an Axiovert Z1 and temperature-controlled incubator (37°C). The LEDs of the Axiovert were run in gated mode to minimise phototoxicity.

2.9. Biochemical methods

2.9.1. Immunobloting

HeLa cells were lysed in 1 x Laemmli buffer (10 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled at 95°C for 5 min. Proteins were resolved on 12 % Tris-Glycine acrylamide gels in 1 x running buffer (25 mM Tris-HCl, 250 mM glycine, 2% SDS) and gels were run at 100 V. Protein were transferred to polyvinylidene difluoride membranes (PVDF, #IPVH00010, MerckMillipore). PVDF membranes were incubated with blocking solution for 1h at RT (75 mM Tris-HCl pH 8.8, 150 mM NaCl, 0.1% Tween20 supplemented with 5% milk). Incubation with the primary

antibody (1 h 30 min) then with secondary goat HRP-conjugated antibodies (1 h) were performed in the blocking buffer at RT. PVDF membranes were washed 3 times for 5 min in blocking solution at RT and developed using PierceTM ECL plus western blotting substrate.

2.9.2. GST-DuD4 purification

Expression and purification of GST-DuD4 was performed as described previously (Schröder *et al*., 2006). *E. coli* BL21 harboring pGEX-4T-FnBPA-Du-D4 was grown at 37°C, and protein synthesis induced with 1 mM isopropyl-β-d-thiogalactopyranoside. Purity of GST-DuD4 protein was analyzed by SDS-PAGE and Western blot by using goat anti-GST (diluted 1:1000; #G7781, Sigma). Protein concentrations were determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA).

2.9.3. Coating of latex beads

1×10^9 latex beads (#LB11, Sigma) were washed and resuspended in 1 ml of sodium borate buffer (boric acid 1M, sodium hydroxide 0.25M, pH 8.5) containing 0.5 mg/ml of GST-FnBPA-Du-D4. The protein was adsorbed to beads overnight at 4°C then beads were washed twice in borate sodium buffer before blocking for 1h with 5 mg/ml bovine serum albumin (BSA) in sodium borate buffer. After incubation, beads were washed and stored at 4°C in borate sodium buffer containing 5 mg/ml of BSA. For co-incubation assays with HeLa cells, abeads-to-cell ratio of 10:1 was used.

2.9.4. Purification of recombinant septins

Recombinant human septins SEPT2–msGFP-SEPT6–SEPT7 complexes were purified as

previously described in (Mavrakis *et al.*, 2014). Plasmids encoding SEPT2 (pnEA-vH-SEPT2), and msGFP-SEPT6 and SEPT7 (pnCS-msGFP-SEPT6–SEPT7) were used. For microscopy-based *in vitro* reconstitution assays, a monomeric superfolder version of green fluorescent protein msGFP tag was fused to the N-terminal of human SEPT6.

Single colonies of *E. coli* BL21 containing plasmids encoding recombinant septins were grown in LB for overnight at 37 ˚C, diluted 1:100 in LB and grown at 37˚C until OD600 0.6-0.8. Afterwards, septin gene expression was induced by adding 1 mM IPTG for 1h. Bacterial cultures were centrifuged at 10 000 x g for 5 min at 4˚C and pellets were lysed in lysis buffer [50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM MgCl2, 10 mM imidazole, 1x protease inhibitor (Roche), 1 mg/ml lysozyme, 1 mM phenylmethylsulfonylfluoride (PMSF)]. Samples were sonicated on ice 8 x 30 sec using 30 % amplitude with 30 sec on ice in between (to maintain protein stability) and centrifuged at 48 000 x g for 30 min at 4˚C. Supernatants were filtered through a 0.45 µm pore filter and placed on lysis buffer equilibrated Ni2+ -NTA columns.

Bound proteins were washed 3 times with washing buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM MgCl2,) and eluted with elution buffer (50 mM Tris, pH 8.0, 500 mM KCl, 500 mM imidazole). In a second purification step, the eluted proteins were passed over equilibrated (50 mM Tris pH 8.0, 300 mM KCl, 5 mM MgCl2) Strep-Tactin columns then bound proteins were washed 3 times with washing buffer (50 mM Tris pH 8.0, 300 mM KCl, 5 mM MgCl2) and eluted with elution buffer (50 mM Tris pH 8.0, 300 mM KCl, 5 mM MgCl2, 2.5 mM desthiobiotin).

2.9.5. *In vitro* assays

S. aureus cultures were grown at 37 °C with shaking at 200 rpm in 5 ml of Hepes minimal medium (50 mM Hepes pH 7,4, 50 mM KCl, 0.5 mM $MgCl_2$, 10 mM $CaCl_2$, 100 mM $MgSO_4$)

salts supplemented with a mix of nutrients (45 µg/ml L-methionine, 20 µg/ml L-tryptophan, 12.5 µg/ml nicotinic acid, 10 µg/ml vitamin B1, 1% glucose, 0.5% casein hydrolysate, 0.1% fatty acid-free BSA). The following day, bacterial cultures were diluted 1:100 in prewarmed M9-Tris-CAA (1:100 v/v) and cultured until an OD_{600} of 0.6. 1.2 ml of bacterial cultures were centrifuged in Low Protein Binding tubes (ThermoFisher Scientific) at 800 × *g* for 2 min at RT and bacterial pellets were resuspended in 100 µl of *in vitro* reconstitution solution (minimal buffer supplemented with 240 nM of septin complex SEPT7/msGFP–SEPT6/SEPT2 and 1 mM dithiothreitol [DTT]). Low Protein Binding tubes containing the sample were placed in opaque conical polypropylene tubes and incubated at 37 °C with shaking at 220 rpm for 2 h. Samples were immediately placed on ice and centrifuged at 800 × *g* at 4 °C for 1.5 min. Bacterial pellets were resuspended in 300 µl of ice-chilled minimal medium and centrifuged at 800 × *g* at 4 °C for 2 min. This step was repeated one more time and pellets were finally resuspended in 100 µl of ice-chilled minimal medium and samples were then used for confocal imaging

2.9.6. Purification of *S. aureus* peptidoglycan sacculi and cell wall

CW and PG fractions were prepared from exponentially growing cultures of *S. aureus* as previously described in (Filipe *et al.*, 2005). Briefly, exponentially grown bacteria were harvested by centrifugation, suspended in ice-cold 50 mM Tris-HCl pH 7.0 buffer and boiled in 4% SDS for 45 minutes to inactivate any CW modifying enzymes and remove contaminating proteins and non-covalently bound lipoproteins. Bacteria were washed several times with water to remove the SDS and CWs were mechanically broken with acid-washed glass beads. Unbroken cells and glass debris were removed by low speed centrifugation and CWs were digested with deoxyribonucleases (DNAses) and ribonucleases (RNAse) for 2 h at 37 °C, then overnight with trypsin at 37 °C to remove DNA, RNA and cell wall bound proteins. CWs were

collected by centrifugation and washed with water before treatment with 8 M LiCl to remove CW glycoproteins and polypeptidic contamination, then with 100 mM EDTA before final treatment with acetone to remove membrane lipids and lipoteichoic acids. The broken CWs were resuspended in water and lyophilized to obtain the “CW fraction”. For purification of PG, the CWs were treated with 49% hydrofluoric acid (HF) for 48h at 4 °C to remove WTA then washed and lyophilised. For preparation of muropeptides the PG was digested with Mutanolysin (Sigma) for high-performance liquid chromatography analysis.

2.10. Statistics

Statistical analysis was performed in Excel (Microsoft, Redmond, Washington, USA) and GraphPad Prism 9.4.1 (GraphPad Software, La Jolla, California, USA). Unless otherwise indicated, data represent the mean ± standard error of the mean (SEM) from at least three independent experiments. A Student’s t-test (two-tailed), one-way or two-way ANOVA were used to test for statistical significance, with p-value < 0.05 considered as significant. All details on statistical analysis (including statistical tests, significance, value of the number of experimental replicates, and bacterial cells quantified) can be found in the figure legends.

Chapter 3. Regulation of integrin α5β1-mediated *S. aureus* cellular invasion by the septin cytoskeleton

3.1. Introduction

3.1.1. Integrin-mediated cell adhesion and cytoskeleton crosstalk

Integrin-mediated ECM adhesions are essential for cell growth, survival, differentiation, and tissue integrity (Bökel and Brown, 2002). Integrins are transmembrane αβ protein heterodimers which sense the extracellular microenvironment to trigger several signalling pathways such as those involved in cell proliferation (Kamranvar *et al.*, 2022). In mammals the integrin proteome contains 18 different α subunits and 8 different β subunits, which can combine to generate at least 24 distinct heterodimers of αβ integrins (Takada *et al.*, 2007). The extracellular domains bind to specific ligands whereas the cytoplasmic tail can interact with a diverse collection of cytoplasmic interactors associated with the actin cytoskeleton (**Figure 6**).

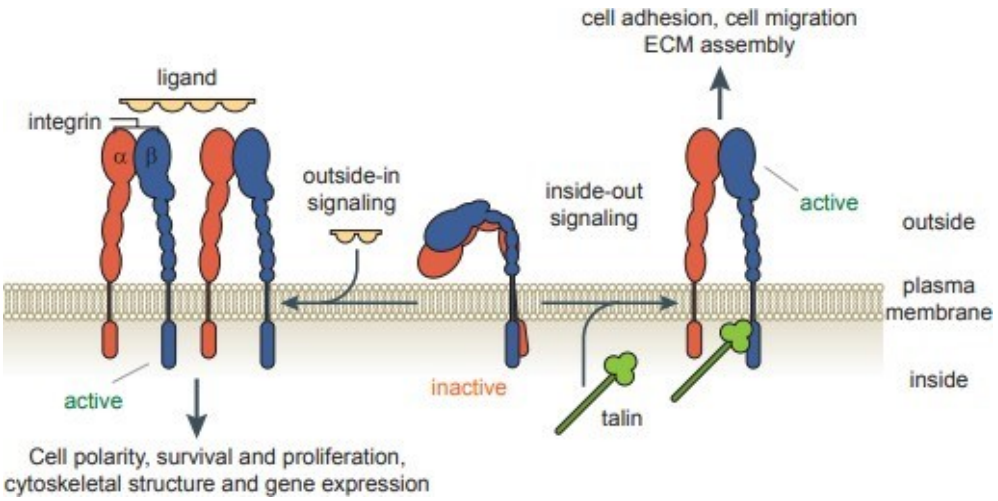

Figure 6. Conformational changes during integrin activation upon ligand binding or by recruitment of a cytoplasmic activator. Adapted from (Shattil *et al.*, 2010)

Activation of integrin upon binding to its ligand (called outside-in signalling) or by recruitment of a cytoplasmic activator (e.g. talin) to the intracellular tail (called inside-out signalling) triggers a conformational change in integrin structure from a bent to an extended state. This conformational change results in activation of downstream signalling pathways.

Members of the family of β1 and β3 integrins have been shown to bind to fibronectin, a major component of the ECM, and localize to FAs (Danen *et al.*, 2002). Cell-matrix adhesions are recognised to grow and mature by a mechanism driven by myosin-II-induced tension (Wu, 2007). Briefly, activated integrins bind to ECM which leads to recruitment of adaptor proteins (such as paxillin and talin) to promote actin polymerisation and form nascent adhesions. Then α-actinin together with myosin II leads to crosslinking actin filaments and formation of contractile actin-filament bundles forming an actomyosin structure and generating focal complexes. The activity of small GTPase Rac1 has also been linked to formation of focal complexes (Guo *et al.*, 2006). The resulting tension leads to recruitment of zyxin and vinculin to promote growth of actomyosin filaments into stress fibers and form mature FAs. In line with this, pharmacological inhibition of actomyosin contractility impairs stability of FAs (Lavelin *et al.*, 2013). Maturation into FAs and actomyosin driven contractility is mediated by the small GTPase RhoA and its effectors Rho kinase and mDia1. Rho kinase stimulates myosin-II activity by inactivating myosin-light-chain phosphatase and phosphorylating myosin II regulatory light chain (MLC), whereas mDia1 promote actin nucleation and polymerisation (Kimura *et al.*, 1996). FAs are believed to finally mature into the centrally located fibrillar adhesions, elongated adhesion structures enriched in α5β1-integrin and tensin (Barber-Pérez *et al.*, 2020). Fibrillar adhesions are stable with a lifetime of several hours and mediate fibronectin remodelling.

A total of 180 distinct proteins collectively termed the integrin ‘adhesome’ have been identified, and although FAs have a conserved nanoscale architecture, this structure is highly

dynamic and the precise molecular mechanisms of adhesion assembly and disassembly are not fully known. Adhesion turnover is regulated by cycles of integrin endocytosis and exocytosis (recycling) to control availability of integrins at the plasma membrane and crosstalk between the different cytoskeletal proteins. Integrin receptor internalisation mechanisms are broadly classified as clathrin-mediated endocytosis or clathrin-independent endocytosis (including caveolin-dependent pathways, micropinocytosis) (Moreno-Layseca *et al.*, 2019). Integrins enter the endo-exocytic cycling pathway, where they can be recycled back to plasma membrane (Paul *et al.*, 2015). Moreover, autophagy targets FA components such as paxillin, driving FA turnover to promote cellular migration (Sharifi *et al.*, 2016). Inhibition of autophagy prevented the lysosomal degradation of internalized β1 integrins and promoted their recycling back to the cell membrane (Tuloup-Minguez *et al.*, 2013).

The cytoskeleton is also involved in FAs disassembly. By using stress fibers as tracks and interacting with KN motif and ankyrin repeat domain-containing protein 1 (KANK1) and talin, microtubules repetitively contact FAs which results in FAs disassembly (Bouchet *et al.*, 2016). Microtubules-induced disassembly requires FA kinase (FAK) and the GTPase dynamin (Ezratty *et al.*, 2005). Moreover, CLIP-associating proteins (CLASP) have been shown to mediate microtubule tethering at FAs, where exocytic vesicles loaded with matrix metalloproteinases (MMPs) will be released to promote ECM degradation, releasing integrin-ECM connections (Stehbens *et al.*, 2014).

Owing to its ability to interact with the actin and microtubule network, as well as the plasma membrane, septins were shown to be important in FAs regulation. In human umbilical vein endothelial (Huvec) cells, septins guide and promote growth of microtubules into FAs to promote their disassembly (Merenich *et al.*, 2022). In murine and human melanoma cells, depletion of SEPT2 or SEPT9 led to the formation of FAs having decreased size (Farrugia *et al.*, 2020), and in cancer-associated fibroblasts, SEPT2 depletion decreased the total number of

FAs (Calvo *et al.*, 2015). In human embryonic kidney (HEK293) cells, overexpression of SEPT2 and SEPT9 led to a significant increase in the number of smaller size FAs (Kang *et al.*, 2021). In contrast to actin and microtubules, high resolution microscopy revealed that septins do not directly contact FAs (Martins *et al.*, 2023), suggesting that the role of septins is mediated by their interaction with the actomyosin stress fibers. In line with this, depletion of septins in cancer-associated fibroblasts and in melanoma cells affected the formation of actomyosin stress fibers (Calvo *et al.*, 2015). In Madin-Darby canine kidney (MDCK) cells where septins are recruited to the lamellar network of stress fibers, depletion of SEPT2 resulted in smaller and short-lived FAs (Dolat *et al.*, 2014).

Together, cell adhesion is a complex process primarily driven by the interplay with microtubules and actomyosin network, and septins are emerging as key players in modulation of FAs maturation and disassembly. Several clinically relevant human pathogens are known to bind integrins and modulate FA signalling to promote invasion into a variety of host cells (Ulanova *et al.*, 2009; Murphy and Brinkworth, 2021).

3.1.2. Integrin α5β1-mediated *S. aureus* cellular invasion

Several *in vitro* and *in vivo* studies have highlighted the importance of FnBPA/B in *S. aureus* invasion and establishment of infection (Speziale and Pietrocola, 2020). It is not fully understood how downstream signalling is transduced but evidence suggests that *S. aureus* co-opt FA signalling to drive cellular invasion (Murphy and Brinkworth, 2021) (**Figure 7**).

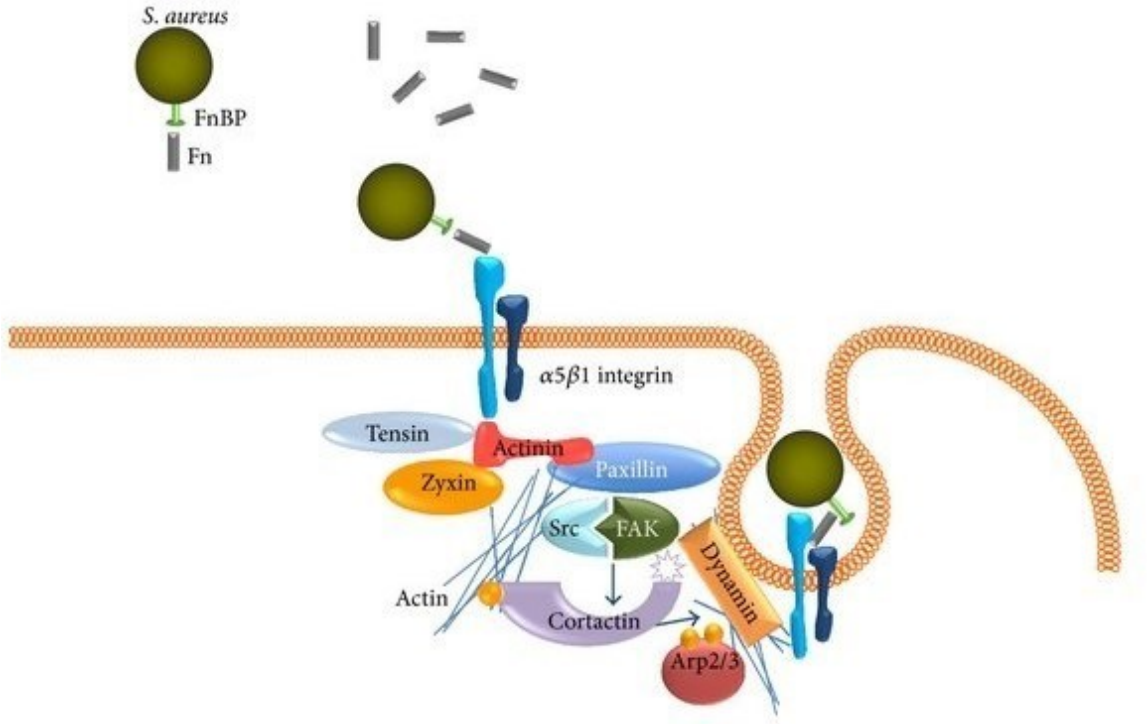

Figure 7. Overview of the FnBP-Fn-integrin α5β1-mediated entry process of *S. aureus* in NPPCs. *S. aureus* expresses FNBPAB to bind host fibronectin and use it as a bridge to activate and clusters host integrin, leading to recruitment of adaptor proteins such as paxillin and actinin and kinases such as FAK, which ultimately leads to actin rearrangements and bacterial internalisation. Adapted from (Alva-Murillo et al., 2014).

In a first step, *S. aureus* FNBPA/B-mediated integrin clustering leads to recruitment of adaptor proteins and FAK which is activated by phosphorylation and serves as a docking site for the proto-oncogene tyrosine-protein kinase (Src). The Src-FAK complex is important for downstream signalling since their silencing reduces *S. aureus* invasion (Agerer *et al.*, 2003; Ji *et al.*, 2020). The active Src-FAK complex phosphorylates the cytoplasmic tail of integrin as well as paxillin and cortactin, which in turn can activate Arp2/3 complex. The Arp2/3 complex will promote actin polymerisation and ultimately the endocytic machinery is engaged to promote internalization of bacteria in a zipper-like process (Schröder *et al.*, 2006). Integrin clustering also promote recruitment of scaffolding proteins such as vinculin, zyxin and integrin-linked kinase (ILK). ILK is a regulator of Cdc42, a mediator of actin remodelling, and both

ILK and Cdc42 have been shown to play a key role in *S. aureus* invasion of NPPCs (Sayedyahossein et al., 2015; Rauch et al., 2016). Additionally, activation of phosphoinositide-3-kinase (PI3K)-Akt pathway, the Extracellular Signal-Related Kinases (ERK) and c-JUN kinases (JNK) pathways appear to be important during *S. aureus* internalization (Ellington *et al.*, 2001; Oviedo-Boyso *et al.*, 2011).

A deeper understanding of the role of the host cytoskeleton during integrin-mediated invasion may help to develop new strategies to control infection. Is it possible to target specific components of the cytoskeleton to interfere with cellular invasion? Given the importance of integrin α5β1 in pathogenesis of *S. aureus* and other bacterial pathogens such as Group A *Streptococcus,* gaining in-depth insights into the signalling underlying integrin-mediated invasion is of critical clinical importance.

In the following results section, we explore a role for the septin cytoskeleton in *S. aureus*-mediated invasion of epithelial cells.

3.2. Results

3.2.1. Investigating a role for the septin cytoskeleton in *S. aureus* invasion

To investigate whether septins play a role in *S. aureus* invasion process we first used the compound UR214-9, a new small molecule inhibitor of septins optimized from the conventional septin inhibitor forchlorfenuron. *In silico* indicated that FCF preferentially interacts with the nucleotide-binding pockets of septins to prevent further GTP binding and hydrolysis (Angelis *et al.*, 2014). UR214-9 displays more optimal docking energies than that of FCF for human septins (Zhovmer *et al.*, 2022). Of note, although FCF has been shown to inhibit septins dynamics both *in cellulo* and *in vitro*, its specificity to septins is still being questioned, thus results UR214-9 should be cautiously interpreted. Unlike forchlorfenuron, UR214-9 cytotoxicity for mammalian cells is reduced and thus suitable for a short term treatment of cells to acutely inhibit septin dynamics (Zhovmer *et al.*, 2021; Zhovmer *et al.*, 2022). In the epithelial cell line MDA-MB-231, UR214-9 has been shown to lead to loss of septin filaments within the first hour of treatment (Zhovmer *et al.*, 2022). We established the optimal concentration of UR214-9 in our experimental system leading to inhibition of septin polymerisation. Treatment of HeLa cells with 50 µM UR214-9 for 30 min led to a collapse of the septin cytoskeleton which accumulated at the perinuclear region (**Figure 8A**).

HeLa cells pretreated 30 min with UR214-9 were infected with *S. aureus* for 30 min and after lysostaphin treatment cells were lysed and plated to determine CFU (**Materials and Methods, section 2.7.4, Figure 5**). Treatment of HeLa cells with UR214-9 abolished *S. aureus* entry (**Figure 8B**). Incubation of *S. aureus* (in the absence of host cells) for 30 min in DMEM + 10% FCS in presence of DMSO or 50 µM UR214-9 showed that UR214-9 does not have bacteriostatic or bacteriolytic action against *S. aureus* (**Figure 8C**). Thus, the decrease in

CFU/ml count is not due to UR214-9-mediated killing of *S. aureus* but to an invasion defect.

These data suggested a potential role of the septin cytoskeleton in *S. aureus* invasion.

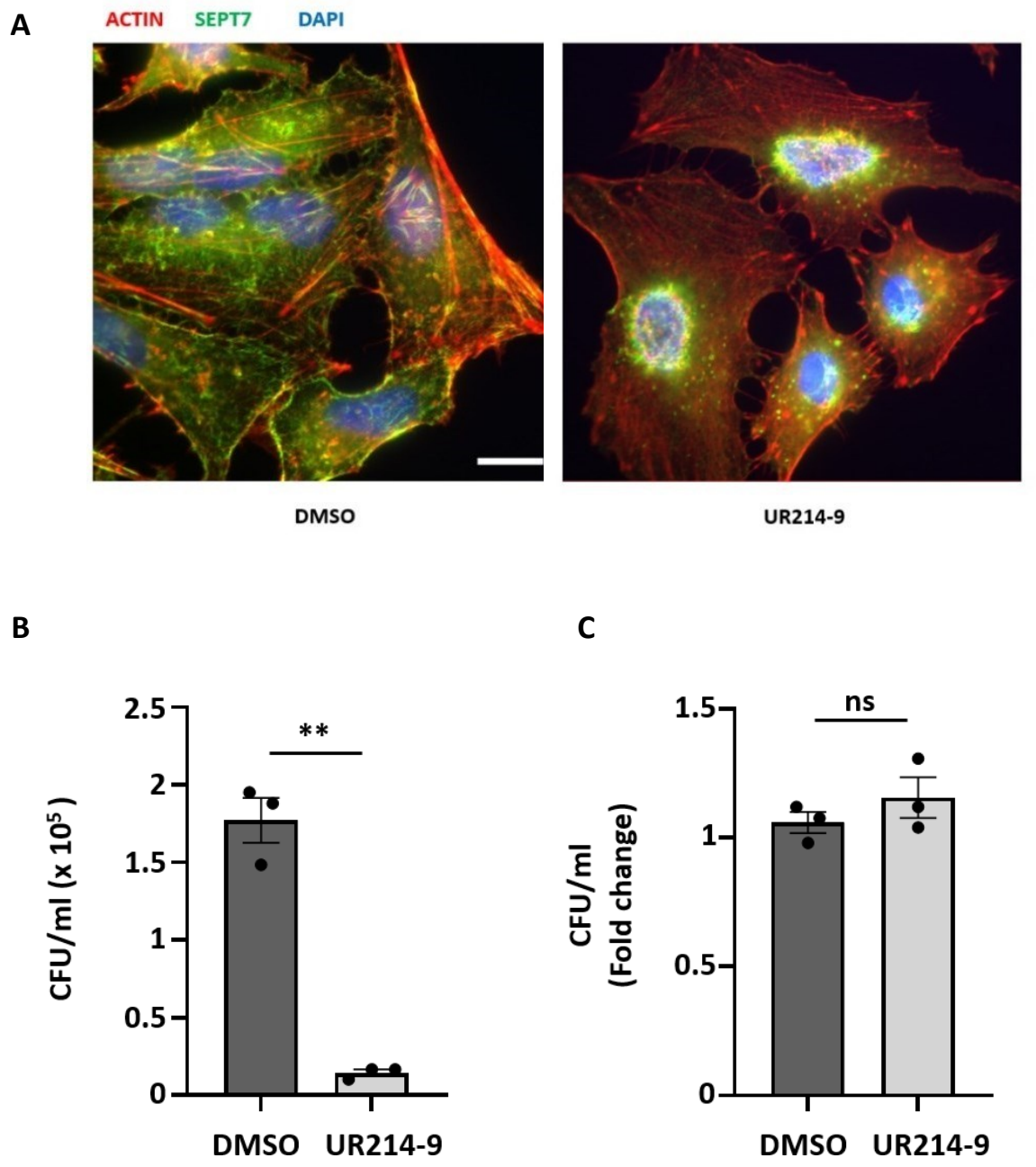

Figure 8. UR214-9 prevents *S. aureus* invasion of HeLa cells and does not have antibacterial properties. A. HeLa cells were treated with DMSO as a control (left) or 50 µM UR214-9 (right) in DMEM + 10% FCS. Cells were stained for SEPT7 (green), F-actin (red) and Hoechst to label DNA (blue). Scale bars represent 15 µm. **B.** HeLa cells were treated with DMSO or 50 µM UR214-9 in DMEM + 10% FCS and infected with *S. aureus*-mcherry at MOI 20 for 30 min to assess invasion. After 10 min lysotaphin treatment cells were lysed and plated on TCS agar plates. The graph represents the mean CFU/ml ± SEM of 3 independent experiments. ** , p-value = 0.008 by Student two-tailed t test. **C.** *S. aureus*-mCherry were treated with either DMSO or with 50 µM of UR214-9 in DMEM + 10% FCS for 30 min. The graph represents the mean fold change in CFU/ml (ratio t=0 / t=30 min) ± SEM of 3 independent experiments. ns: non-significant by Student two-tailed t test.

3.2.2. Purification of GST-DuD4 and coating of beads

Given the importance of the FnBPA/B-Fn-α5β1integrin pathway in *S. aureus* invasion, we purified recombinant GST-DuD4 (DuD4 are the domains of FnBPA which specifically bind fibronectin) (**Figure 9A**). By using GST-DuD4 coated latex beads of 1 µm diameter, this will allow us to study a potential role for septins in FnBPA-mediated invasion in the absence of other bacterial co-factors. To test the functionality of our purified protein, coated latex beads (prepared as indicated in **Materials and Methods, section 2.9.3.**) were co-incubated with HeLa cells in DMEM + 10% FBS for 15 min then cells were fixed and immunostained to detect GST and activated integrin α5β1. We observed clear enrichment of activated integrin α5β1 to GST-DuD4 coated beads showing that the coating process was efficient and the purified proteins are functional (**Figure 9B**).

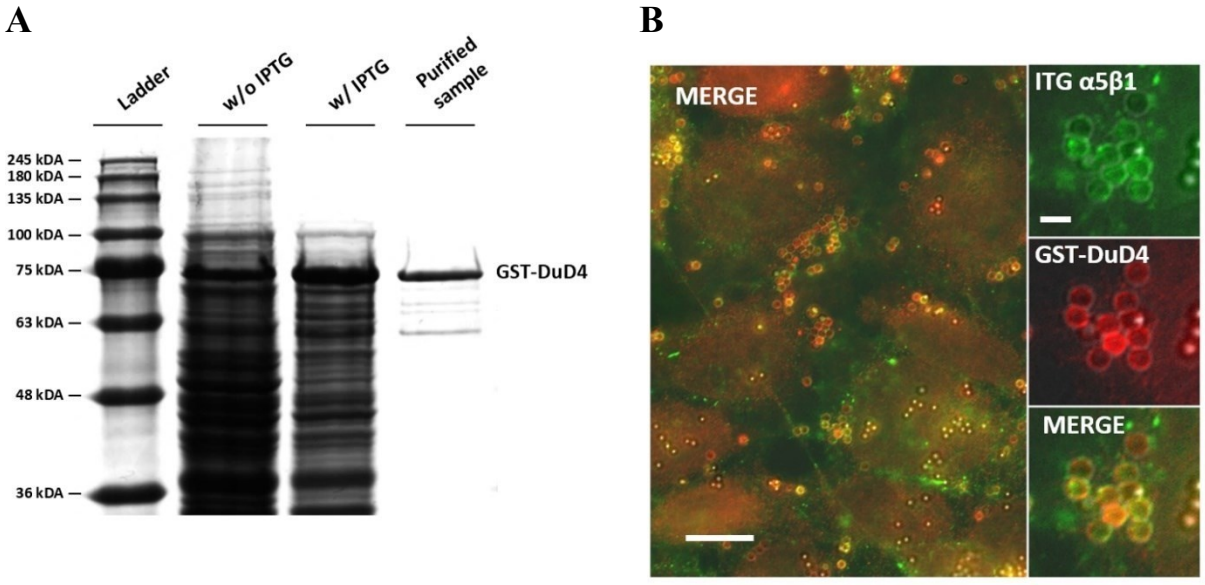

Figure 9. Purification of GST-DuD4 and coating of latex beads of 1 µm diameter. **A.** Coomassie straining showing purification of GST-DuD4 from *E. coli* bearing a vector with IPTG-inducible expression of GST-DuD4 (*E. coli* GST-DuD4). Lane 1 : ladder; Lane 2: lysate of *E. coli* GST-DuD4 without IPTG; Lane 3 : lysate of *E. coli* GST-DuD4 with 1 mM IPTG induction; Lane 4 : purification sample. **B.** HeLa cells were challenged with GST-DuD4-coated beads of 1 µm diameter for 15 min in DMEM + 10% FBS. After fixation, cells were stained for GST (red) and active integrin α5β1 (ITGA5B1, green). DIC microscopy (grey) is shown merged with each channel to visualize the beads. Scale bars are 15 µm (main image) and 2 µm (inset).

In the following section, we investigated a role for septins in the FnBPAB-Fn-integrin α5β1-mediated entry process of *S. aureus* which has been shown to be crucial for establishment of infection *in vivo* (Speziale and Pietrocola, 2020).

3.2.3. Publication : Regulation of integrin α5β1-mediated *S. aureus* cellular invasion by the septin cytoskeleton

The content of this section is published.

Robertin, S., Brokatzky, D., Lobato-Márquez, D., & Mostowy, S. (2023). Regulation of integrin α5β1-mediated *Staphylococcus aureus* cellular invasion by the septin cytoskeleton. European journal of cell biology, 102(4), 151359. https://doi.org/10.1016/j.ejcb.2023.151359

RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.

SECTION A – STUDENT DETAILS

STUDENT ID NUMBER	LSH1806498	**TITLE**	MR
FIRST NAME(S)	Stevens Eugene		
SURNAME/FAMILY NAME	Robertin		
THESIS TITLE	Investigating New Roles for Septins in Host-Pathogen Interactions Using Staphylococcus aureus		
PRIMARY SUPERVISOR	Serge Mostowy		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

<u>SECTION B – Paper already published</u>

Where was the work published?	European Journal of Cell Biology		
When was the work published?	September 2023		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	**Yes**

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	The paper was written by me, Stevens Robertin, with the help of my supervisor Serge Mostowy. All experiments were performed by myself except those involving flow cytometry and qRT-PCR (samples were prepared by me, flow cytometry analysis and qRT-PCR were performed by Dr Dominik Brokatzky) and purification of GST-DuD4 was performed with the help of Dr Damián Lobato-Márquez.

SECTION E

STUDENT SIGNATURE	STEVENS ROBERTIN
DATE	14/10/2023

SUPERVISOR SIGNATURE	SERGE MOSTOWY
DATE	14/10/2023

Regulation of integrin α5β1-mediated *Staphylococcus aureus* cellular invasion by the septin cytoskeleton

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Keywords

cytoskeleton, integrin, septins, *Staphylococcus aureus*

Abstract

Staphylococcus aureus, a Gram-positive bacterial pathogen, is an urgent health threat causing a wide range of clinical infections. Originally viewed as a strict extracellular pathogen, accumulating evidence has revealed *S. aureus* to be a facultative intracellular pathogen subverting host cell signalling to support invasion. The majority of clinical isolates produce fibronectin-binding proteins A and B (FnBPA and FnBPB) to interact with host integrin α5β1, a key component of Fas. *S. aureus* binding of integrin α5β1 promotes its clustering on the host cell surface, triggering activation of FA kinase (FAK) and cytoskeleton rearrangements to promote bacterial invasion into non-phagocytic cells. Here, we discover that septins, a component of the cytoskeleton that assembles on membranes, are recruited as collar-like structures with actin to *S. aureus* invasion sites engaging integrin α5β1. To investigate septin recruitment to the plasma membrane in a bacteria-free system, we used FnBPA-coated latex beads and showed that septins are recruited upon activation of integrin α5β1. SEPT2 depletion reduced *S. aureus* invasion, but increased surface expression of integrin α5 and adhesion of *S. aureus* to host cells. Consistent with this, SEPT2 depletion increased cellular protein levels of integrin α5 and β1 subunits, as well as FAK. Collectively, these results provide insights into regulation of integrin α5β1 and invasion of *S. aureus* by the septin cytoskeleton.

Introduction

Staphylococcus aureus is a major human bacterial pathogen associated with nosocomial infections, which can lead to life threatening conditions such as acute endocarditis and bacteraemia (Tong et al., 2015). Initially classified as a strict extracellular pathogen, several studies have shown that some strains of *S. aureus* can invade and survive inside a wide variety of non-phagocytic host cells *in vitro* and *in vivo* (Watkins and Unnikrishnan, 2020, Rodrigues Lopes et al., 2022). Consistent with this, cellular invasion by *S. aureus* is associated with recurrent rhinosinusitis (Clement et al., 2005), tonsillitis (Zautner et al., 2010) and chronic osteomyelitis (Dongqing et al., 2018). *S. aureus* can thus be viewed as a facultative intracellular pathogen that can subvert host cell signalling to support invasion into host cells (Soe et al., 2021).

The invasion of *S. aureus* into non-phagocytic cells is promoted by a "zipper-type" mechanism, involving F-actin rearrangements (Speziale and Pietrocola, 2020). *S. aureus* invasion into host cells is mediated by the two adhesin fibronectin-binding proteins A and B (FnBPA and FnBPB). FnBPA possesses several fibronectin-binding regions which allows it to interact with fibronectin molecules and use them as a bridge to bind and activate integrin α5β1 (Sinha et al., 1999, Edwards et al., 2010). Previous work showed that preincubated cells with blocking anti-integrin α5β1 antibodies reduced *S. aureus* invasion by > 50%, whereas preincubation with blocking anti-integrin alpha V and alpha V beta3 (other fibronectin-binding integrins) antibodies did not significantly impact *S. aureus* invasion (Sinha et al., 1999). Integrin α5β1 is a heterodimeric cell surface receptor consisting of an α and β subunit, which can be found in either an active or inactive conformation (Hynes, 2002). Integrin α5β1 is typically found in cell-matrix adhesion structures, including both focal and fibrillar adhesions (Kanchanawong and Calderwood, 2023). In the case of *S. aureus* infection, binding to integrin α5β1 results in

its clustering on the host cell surface, triggering FA signalling pathways and recruitment of actin to promote bacterial invasion (Schröder et al., 2006). Several studies have highlighted the importance of FnBP-mediated invasion *in vivo*. For example, in a rat model of endocarditis the expression of *S. aureus* FnBPs in *Lactococcus lactis* is critical for valve colonization (Que et al., 2005), and in a mouse model of mastitis the expression of FnBPs promotes *S. aureus* colonization of mammary glands (Brouillette et al., 2003). The intracellular niche protects *S. aureus* from phagocytosis by macrophages or neutrophils and from extracellular antibiotics. Therefore, studying the internalisation of *S. aureus* by non-phagocytic cells can provide fundamental insights regarding the prevention and treatment of *S. aureus* infection.

Septins are evolutionarily conserved GTP-binding proteins that associate with cellular membranes, actin filaments and microtubules (Woods and Gladfelter, 2021, Spiliotis and Nakos, 2021). Septins from different subgroups interact with each other to form hexameric (SEPT2-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2) or octameric (SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-SEPT2) complexes that assemble end-to-end into nonpolar filaments and higher-order structures (e.g., bundles, rings, cage-like structures) to perform cellular functions such as cytokinesis and phagocytosis (Robertin and Mostowy, 2020). Septins can interact with actomyosin networks which play key roles in mechanotransduction and cell migration (Lam and Calvo, 2019). In the case of bacterial invasion of human cells, work has shown that septins regulate entry of *Listeria monocytogenes, Shigella flexneri, Salmonella enterica Typhimurium and Pseudomonas aeruginosa* (Aigal et al., 2022, Boddy et al., 2018, Mostowy et al., 2009, Mostowy and Cossart, 2012). Despite over a decade of investigation, the precise role of septins in bacterial invasion of host cells is poorly understood. In this study we discover a new role for septins in regulation of integrin α5β1 and *S. aureus* invasion.

Results

Septins are recruited to the invasion site of *S. aureus*

To investigate the role of septins during *S. aureus* invasion, HeLa cells were infected with *S. aureus* strain SH1000 producing cytosolic mCherry for 15 min. Following fixation, infected cells were labelled for SEPT7 and F-actin (used as a marker of invasion sites). We observed that SEPT7 is recruited as a collar-like structure to ~98% of *S. aureus* present at actin-rich invasion sites (Fig. 1A, B). Reconstructed XZ-confocal cross-section images revealed that septins accumulated along extending protrusions of the actin-rich invasion site (Fig. 1C). SEPT2 and SEPT9 are similarly recruited to *S. aureus* invasion sites, suggesting that octameric complexes composed of SEPT2-SEPT6-SEPT7-SEPT9 are involved (Suppl Fig. 1A). Inhibition of actin polymerization has been shown to restrict *S. aureus* invasion (Agerer et al., 2005). Treatment of cells with cytochalasin D prevented recruitment of both actin and SEPT7 to invading bacterial cells (Suppl Fig. 1B), suggesting that septin recruitment is dependent on actin. Septins can be associated to actin filaments by binding non-muscle myosin II (myosin II), and this association is important for myosin II activation (Joo et al., 2007). We stained for phosphorylated myosin light chain IIA (pMLC), a marker of myosin II activation, to test for recruitment of pMLC with septins at *S. aureus* invasion sites. We observed the recruitment of pMLC to septin-associated bacteria, and that inhibition of myosin II activity using blebbistatin restricted *S. aureus* entry (Suppl. Figs. 1C and 1D). Work has shown that (after internalization) the *S. aureus* containing vacuole is positive for early endosomal markers including the small GTPase Rab5 (Schröder et al., 2006). We exploited the association of Rab5 to follow the internalization process of *S. aureus* in live cells. To visualize the dynamic recruitment of septins and actin during *S. aureus* invasion, we infected Hela cells stably producing GFP- SEPT6 and iRFP-LifeAct, and transiently expressing mCherry-Rab5 (Fig. 1D). In this case, we

observed that GFP-SEPT6 and iRFP-LifeActin are synchronously recruited to invading bacteria. The recruitment of GFP-SEPT6 and iRFP-LifeAct preceded the recruitment of mCherry-Rab5 (Fig. 1E), further supporting our conclusion that septins are involved at an early stage of the invasion process.

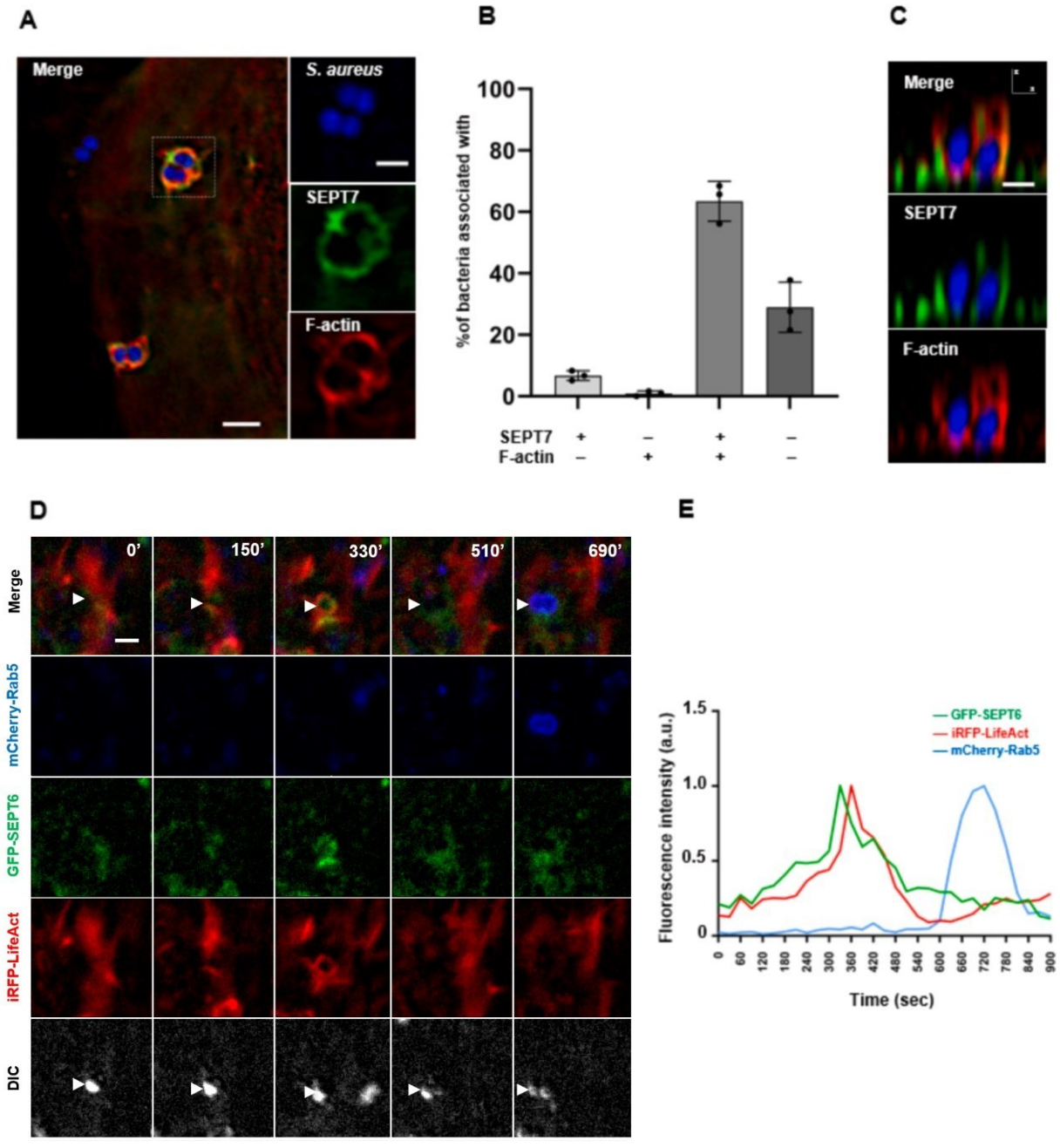


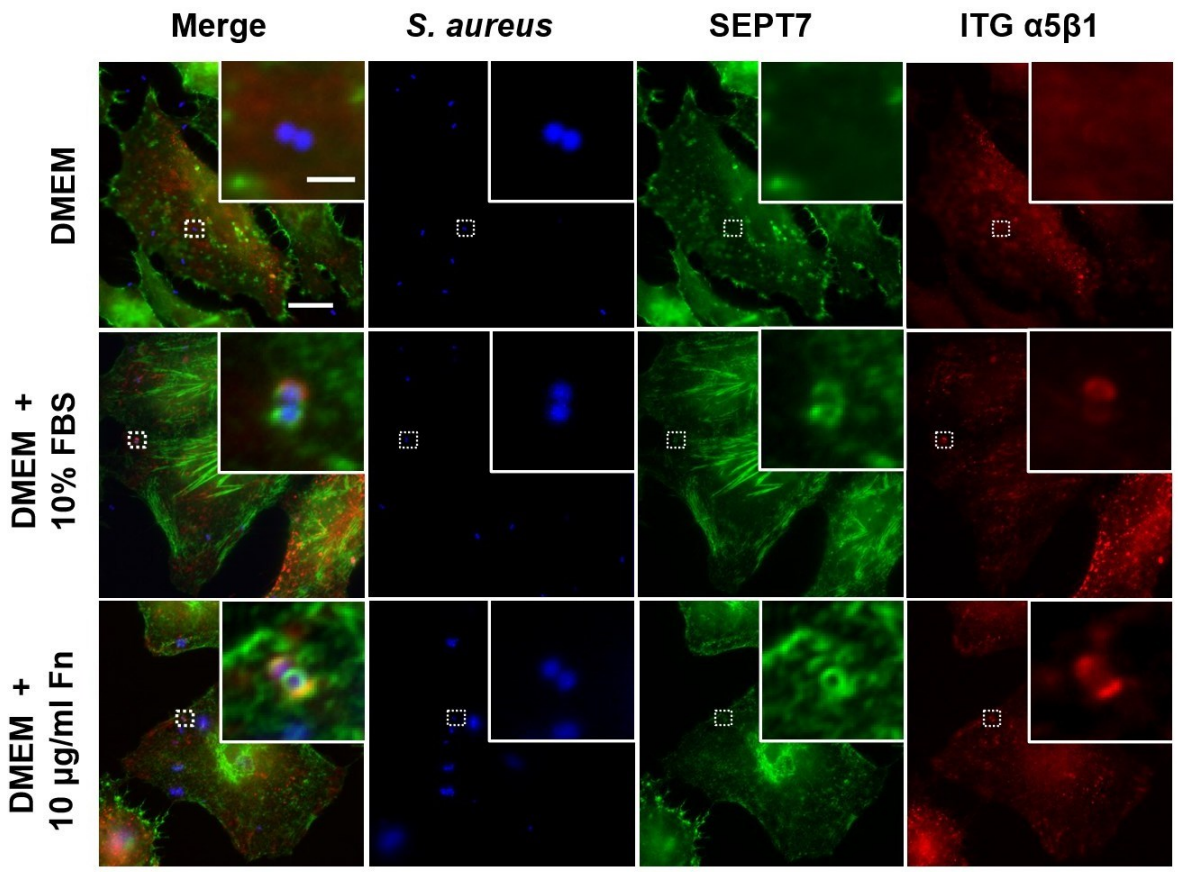
Fig. 1. Septins are recruited to the invasion site of *S. aureus*. (A) HeLa cells were infected with mCherry-*S. aureus* SH1000 (blue) at MOI of 20 in DMEM + 10% FBS and fixed 15 min post invasion. Cells were stained for SEPT7 (green) and F-actin (red). Scale bars represent 2 µm (main image) and 1 µm (inset). (B) Quantification of the percentage of bacteria colocalizing with SEPT7 and/or F-actin from (A). The graph represents mean percentage

± SEM from of n = 517 bacteria from three independent experiments. (C) Reconstructed XZ-confocal cross-section images data of the invading bacteria from inset in 1 A. Bacteria are shown in blue, F-actin in red and SEPT7 in green. Scale bar represents 1 µm. (D) Time-lapse of the association of GFP-SEPT6 (green), mCherry-Rab5 (blue), and LifeAct-iRFP (red) with *S. aureus* in infected HeLa cells in DMEM + 10% FBS. "Merge" indicates the merged channels excluding DIC. The time in seconds is shown. DIC microscopy is shown to visualize bacteria. Arrowheads indicate the position of bacteria. Scale bar represents 2 µm. (E) Normalized fluorescence intensity of each protein recruited to *S. aureus* over time during invasion event illustrated from Fig. 1D. Images were acquired every 30 s, intervals of 60 s are indicated on x axis. Loss of GFP-SEPT6 and LifeAct-iRFP coincides with the recruitment of mCherry-Rab5.

Septins are associated with integrin α5β1-mediated internalisation of *S. aureus*

Invasion of non-phagocytic cells by *S. aureus* is mainly mediated by adhesins FnBPA and FnBPB which bind to fibronectin and activate integrin α5β1 (Speziale and Pietrocola, 2020). To test if septins are recruited to bacteria in an integrin α5β1-dependent manner, HeLa cells were infected with mCherry-*S. aureus* for 15 min. Following fixation, infected cells were labelled for SEPT7 and the active (ligand bound) conformation of integrin α5β1. In the absence of fibronectin (DMEM, negative control), neither SEPT7 nor integrin α5β1 are recruited to bacteria (Fig. 2A and 2B). In contrast, septins are recruited to ~92% of bacteria activating integrin α5β1 in the presence of 10% fetal bovine serum (FBS, serum naturally contains fibronectin) or in the presence of 10 µg/ml purified fibronectin (Fig. 2A and 2B). Upon integrin α5β1 activation, FA kinase (FAK) undergoes autophosphorylation and triggers downstream signaling (Agerer et al., 2003). In agreement with a role for septins in integrin α5β1 mediated invasion, we observed that SEPT7 is recruited to invasion sites with phosphorylated active FA kinase (pFAK-Y397) (Fig. 2C). Together, these data suggest that septins may be involved in integrin α5β1 mediated invasion of *S. aureus*.

A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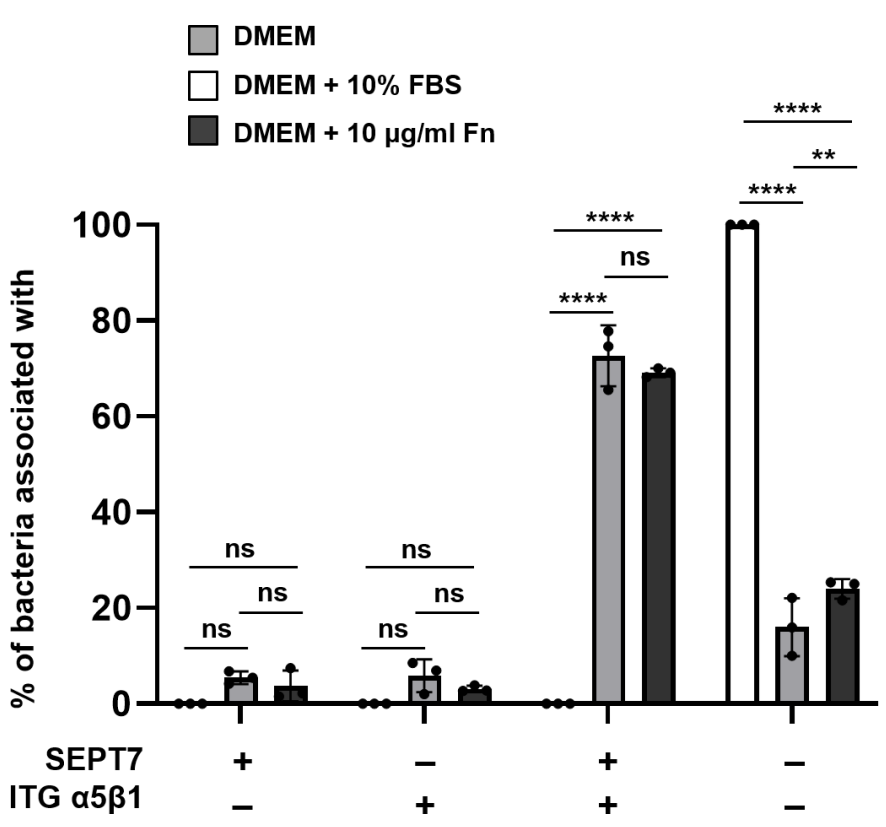
S. aureus
SEPT7
ITG α5β1
DMEM
DMEM + 10% FBS
DMEM + 10 µg/ml Fn

B


DMEM
DMEM + 10% FBS
DMEM + 10 µg/ml Fn
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100
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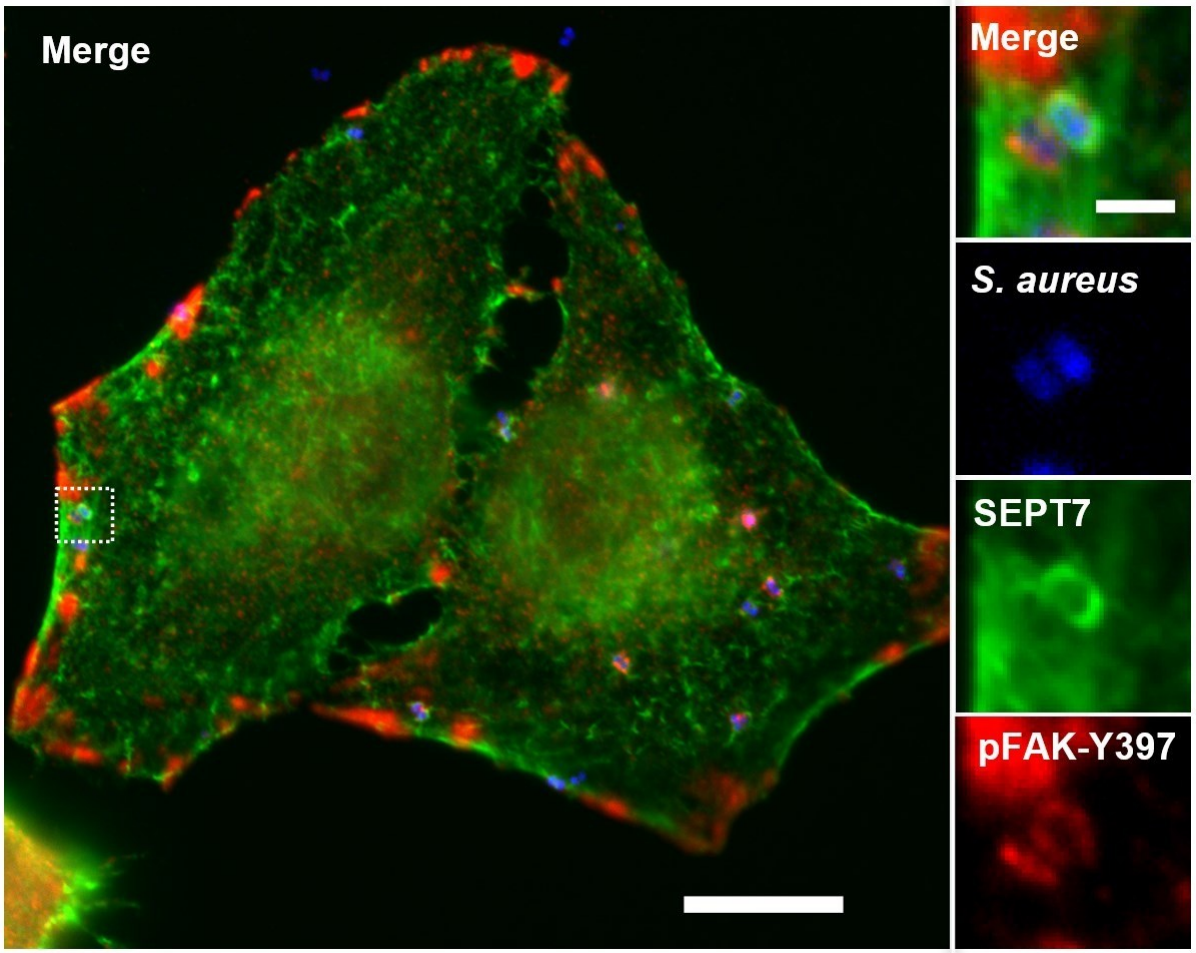
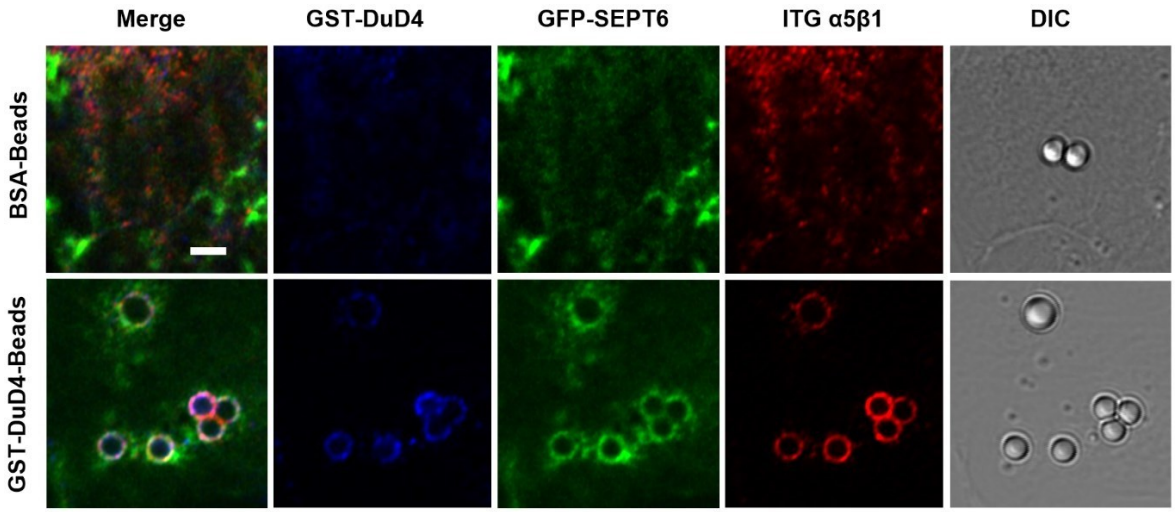


Fig. 2. Septins are associated with integrin α5β1-mediated internalisation of *S. aureus*. (A) HeLa cells were infected with mCherry-*S. aureus* (blue) for 15 min in DMEM either non-supplemented, supplemented with 10% FBS or supplemented with 10 µg/ml purified bovine plasma fibronectin (Fn). After fixation, cells were stained for SEPT7 (green) and active integrin α5β1 (ITG α5β1, red). Scale bars represent 10 µm (main image) and 2 µm (inset). (B) Quantification of (A). Percentage of bacteria localizing with SEPT7 and/or active integrin α5β1 (ITG α5β1) in presence of DMEM only, DMEM + 10% FBS or DMEM + 10 µg/ml Fn. The graph represents mean percentage ± SEM of n= 235 (DMEM), n = 682 (DMEM + 10% FBS) and n = 724 (DMEM + 10 µg/ml purified fibronectin) bacteria from three independent experiments. ns: non-significant; **, p-value = 0.0085; **** p-value < 0.0001 by two-way Anova and Tukey's post-test. (C) Septins are associated with bacteria recruiting phosphorylated FAK (pFAK-Y397). HeLa cells were infected with mCherry-*S. aureus* (blue) for 15 min in DMEM + 10% FBS. After fixation, cells were stained for SEPT7 (green) and pFAK-Y397 (red). Scale bars are 10 µm (main image) and 1 µm (inset).

FnBPA is sufficient to trigger septin recruitment to invading beads

Previous work has shown that polystyrene beads coated with the DuD4 fragment of *S. aureus* FnBPA (that harbors most of the fibronectin-binding repeats) bind integrin α5β1 and triggers formation of fibrillar adhesion-like attachment sites, resulting in the internalisation of beads by non-phagocytic cells (Sinha et al., 1999). To test whether FnBPA-mediated activation of integrin α5β1 is sufficient to trigger septin recruitment, we employed a bacteria-free system where HeLa cells stably producing GFP-SEPT6 were inoculated with BSA-coated (as a control) or GST-DuD4-coated beads of 1 µm in diameter (i.e. approximately the same diameter as *S. aureus*). Here, we observed that BSA coated beads bound non-specifically to cells and did not activate integrin α5β1 nor recruit GFP-SEPT6. In contrast, we observed that GFP-SEPT6 is recruited as collar-like structures to GST-DuD4 coated beads shown to activate integrin α5β1 (Fig. 3A). Using HeLa cells we also observed SEPT7 recruitment to GST-DuD4 coated beads and quantifications showed that SEPT7 is recruited to ~44% of GST-DuD4 coated beads activating integrin α5β1 (Fig. 3B). These data demonstrate that, via fibronectin binding, FnBPA mediated activation of integrin α5β1 is sufficient to trigger septin recruitment to invading beads.

A



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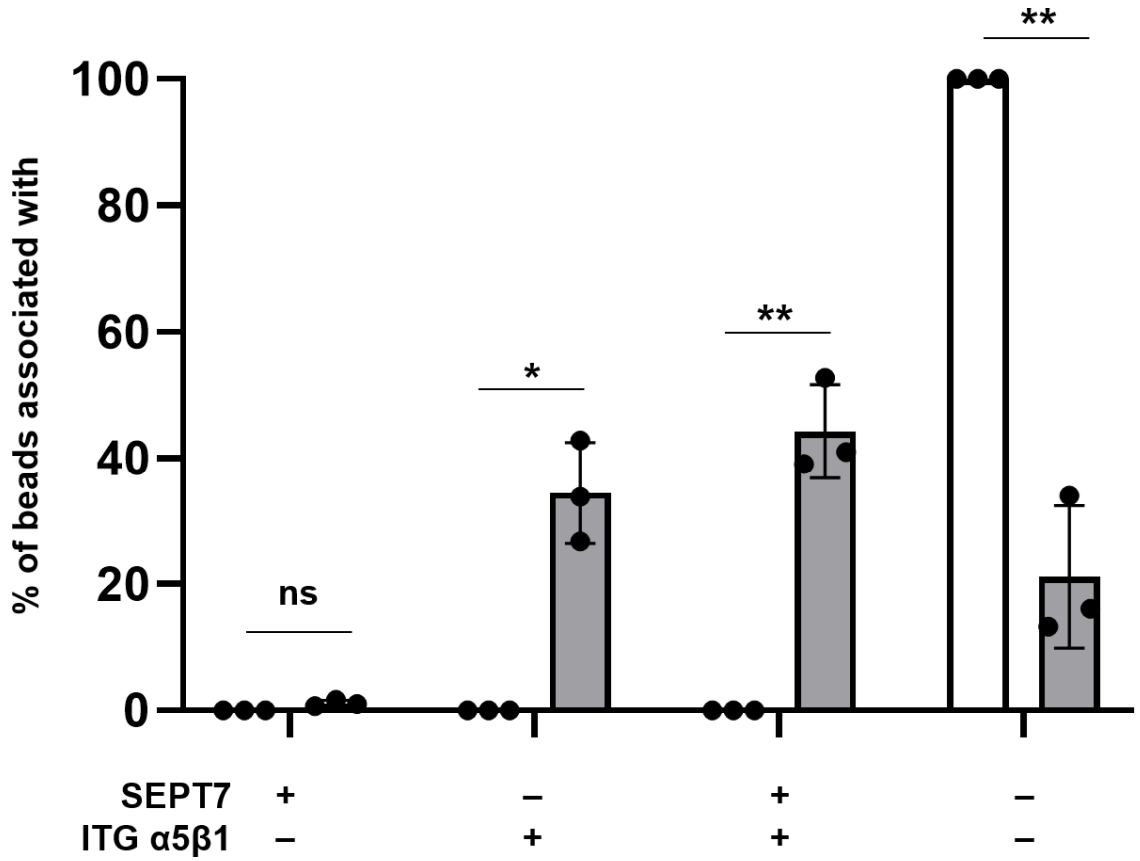

Fig. 3. FnBPA is sufficient to trigger septin recruitment to invading beads. (A) HeLa cells stably producing GFP-SEPT6 were challenged with BSA-coated or GST-DuD4-coated beads of 1 µm diameter for 15 min in DMEM + 10% FBS. After fixation, cells were stained for GST (blue) and active integrin α5β1 (ITG α5β1, red). "Merge" indicates the merged channels excluding DIC. DIC microscopy is shown to visualize the beads. Scale bar represents 2 µm. (B) HeLa cells were challenged with BSA-coated and GST-DuD4 coated beads of 1 µm diameter for 15 min in DMEM + 10% FBS. Quantification of the mean percentage of beads localizing with SEPT7 and/or active integrin α5β1 (ITG α5β1) from (A). n = 191 beads (BSA) and n = 647 beads from three independent experiments. ns : non-significant, * p-value = 0.02, ** p-value < 0.01

Septin depletion impairs cellular invasion by *S. aureus*

Septins have previously been shown to promote internalisation of bacterial pathogens into mammalian epithelial cells (Mostowy et al., 2009, Robertin and Mostowy, 2020). To determine if septins regulate α5β1-mediated *S. aureus* invasion, HeLa cells were treated with siRNA

targeting SEPT2, SEPT7 or SEPT9 (Suppl. Fig. 2A, B). The depletion of SEPT2 and SEPT7 significantly reduced *S. aureus* invasion as compared to control cells (respectively 0.5-fold, p-value = 0.001; 0.7-fold, p-value = 0.049) (Fig. 4A). In contrast, the depletion of SEPT9 did not significantly impact *S. aureus* invasion (Fig. 4A). In agreement with reduced invasion, SEPT2 depletion reduced the percentage of bacteria associated with actin (0.5-fold, p-value = 0.03) (Fig. 4B). Moreover, the percentage of bacteria associated with active integrin α5β1 and phosphorylated FAK was also reduced (respectively 0.7-fold, p-value = 0.006; 0.6-fold, p-value = 0.008) (Suppl. Fig. 2C, D). Unexpectedly, we noticed that SEPT2 depletion significantly increased the number of bacteria associated with the surface of host cells (Fig. 4C). Flow cytometry analysis revealed a significant increase (1.3-fold, p-value = 0.03) in surface expression of integrin α5 and a non-significant increase (1.3-fold, p-value = 0.07) in surface expression of integrin β1, in SEPT2-depleted cells as compared to control cells (Fig. 4D). Moreover, western blot analysis revealed that, as compared to control cells, SEPT2-depleted cells increased protein levels of integrin α5 and β1 subunits, as well as FAK (Fig. 4E, F). By comparison, SEPT7 and SEPT9 depletion did not significantly affect protein levels of integrin α5 and β1 subunits (Suppl. Fig. 2E, F). We performed RT-qPCR using RNA extracted from cells treated with CTRL or SEPT2 siRNA to assess expression of genes encoding for integrin α5 (itga5), β1 (itgb1) or FAK (ptk2). These data showed that SEPT2 depletion did not significantly change expression of *itga5, itgb1* and *ptk2* (Suppl. Fig. 2G). Together, we conclude that SEPT2 regulates integrin α5β1-mediated invasion of *S. aureus.*

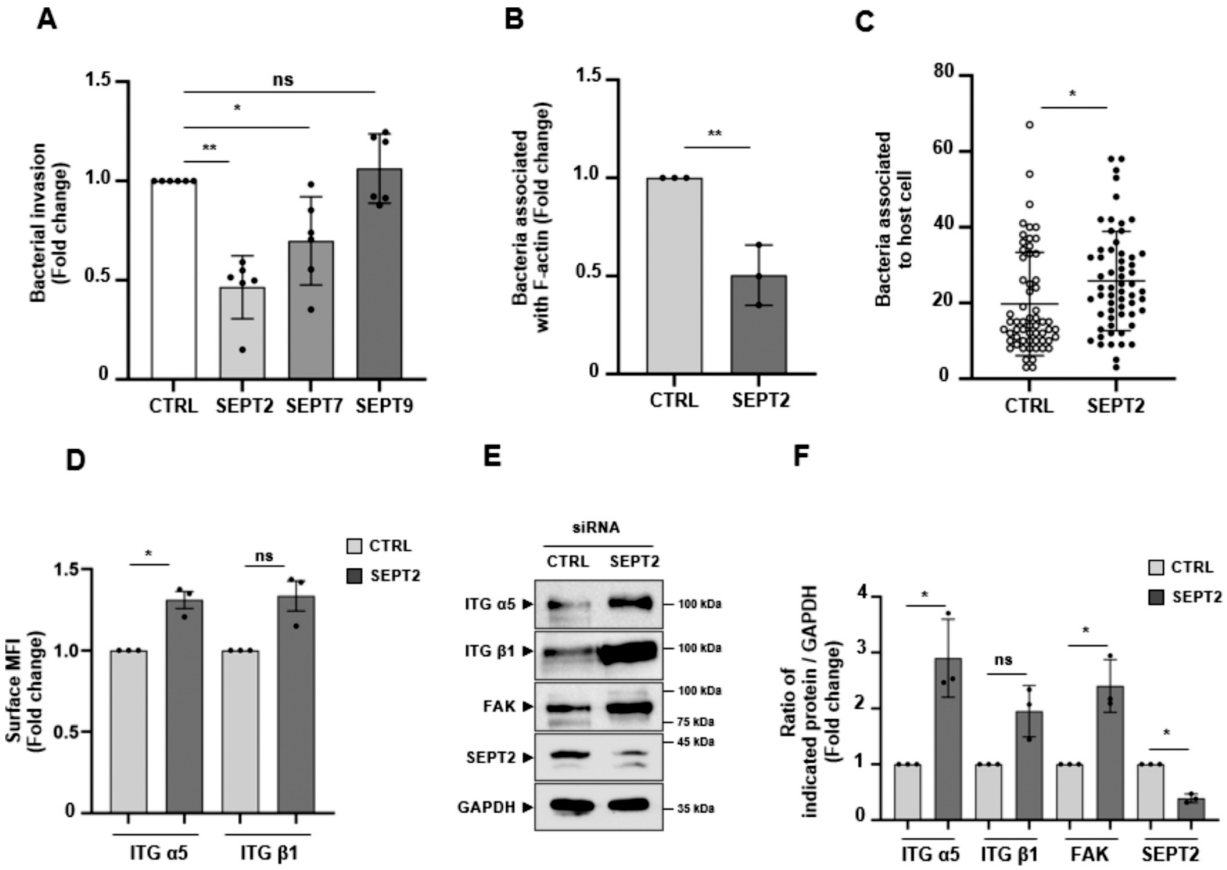

Fig. 4. SEPT2 depletion impairs cellular invasion by *S. aureus*. (A) HeLa cells were transfected with the indicated siRNAs for 72 h. Post-siRNA transfection, cells were infected with *S. aureus* for 30 min in DMEM+10% FBS for gentamicin-lysostaphin protection assays then lysed and plated on TCS agar plates. The graph represents the CFU normalized to the control siRNA treated samples. The CFU ± SEM from 6 independent experiments is presented. * , $p < 0.05$; **, $p < 0.01$; ns: non-significant by one-way Anova and Dunnett's post-test. (B) HeLa cells were transfected with the indicated siRNAs for 72 h. Post-siRNA transfection, cells were infected with *S. aureus* for 15 min in DMEM +10% FBS. After thorough washing steps (no gentamicin-lysostaphin incubation step) cells were fixed and stained for SEPT7 and F-actin. The graph represents mean percentage ±SEM normalized to cells treated with control siRNA and SEM of bacteria associated with F-actin from n = 828 (CTRL siRNA) or n = 1080 (SEPT2 siRNA) bacteria in three independent experiments. * *, $p < 0.01$ by two-tailed Student's t-test. (C) Number of bacteria per cell ± SEM from the same set of images analysed for Fig. 4B. Each dot represents a single cell (n = 60 cells from three independent experiments). * , $p < 0.05$ two-tailed by Student's t-test. (D) Flow cytometry analysis of integrin α5 and β1 surface expression of HeLa cells treated for 72 h with the indicated siRNAs. The graph represents the mean fluorescence intensity (MFI) normalized to cells treated with control siRNA ± SEM from 3 independent experiments. Data is normalised to cells treated with control siRNA. * , $p < 0.05$; ns: non-significant by two-tailed Student's t-test. I Western blot for siRNA depletion experiments corresponding to data presented in Fig. 4B-D. Whole-cell lysates were from control siRNA or SEPT2 siRNA treated HeLa cells. Integrin α5 (ITG α5), integrin β1 (ITG β1), FAK (FAK) and SEPT2 (SEPT2, to show depletion efficiency) protein levels were detected. GAPDH protein level was used as a loading control. (F) Densitometry-based quantification of integrin α5 (ITG α5), integrin β1 (ITG β1), FAK and SEPT2 signal frI(E). All bands were first normalized to the signal of the loading control GAPDH. Graph represents the fold change of the indicated protein normalised to control siRNA treated cells (CTRL) ± SEM from 3 independent experiments. * , $p < 0.05$, ns: non-significant by two-tailed S'udent's t-test.

Discussion

In this study we reveal that septins are involved in the invasion of *S. aureus* into host cells. We show that septins are recruited as collar-like structures with actin at the invasion site. Staining for endogenous SEPT2, SEPT7 and SEPT9 indicated that these different septins are all present at *S. aureus* invasion sites, suggesting involvement of the octameric septin complex SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-SEPT2. Considering that septins sense membrane curvature at the micron-scale (Bridges et al., 2016), it is possible that septins are recruited to *S. aureus* invasion sites because of membrane curvature presented by the phagocytic cup (Lobato-Márquez and Mostowy, 2016). At the invasion site, septins may act as scaffolding proteins or diffusion barriers to coordinate a local response promoting bacterial invasion.

While septins are increasingly being associated to FAs (Lam and Calvo, 2019, Farrugia et al., 2020) and bacterial pathogenesis (Mostowy and Cossart, 2012, Robertin and Mostowy, 2020), our study is the first to reveal a role for septins in integrin α5β1-mediated bacterial invasion. FAs are integrin rich signalling platforms that link the ECM to the cytoskeleton, where actomyosin networks play an important role. In transformed renal epithelial cells, septins interact with actomyosin networks that modulate FA maturation (Dolat et al., 2014). In fibroblasts, SEPT2 depletion altered formation of actomyosin stress fibers and FAs (Calvo et al., 2015). Septins have been shown to colocalize with stress fibers in U2OS cells, but in this case were excluded from FAs (Martins et al., 2023). Physical properties of the ECM (i.e., 3D vs 2D substrates) can govern cell adhesion and mechanotransduction. For example, 3D-matrix adhesions differ from focal and fibrillar adhesions characterized on 2D substrates in their recruitment of integrin α5β1, paxillin and other cytoskeletal components (Cukierman et al., 2001). Considering this, and that *S. aureus* or beads can be viewed as a 3D substrate for

fibronectin, in this work we may be inducing recruitment of proteins which may not be observed when integrin binds fibronectin on a 2D substrate. Further work is required to understand how physical and mechanical parameters may modulate septin recruitment.

Our study shows that depletion of SEPT2 or SEPT7 (but not SEPT9) reduces invasion of *S. aureus* into HeLa cells, and SEPT2 depletion reduces the percentage of bacteria associated with actin, integrin α5β1 and phosphorylated FAK. Previous work has shown that SEPT2, SEPT6, SEPT7, SEPT9 and SEPT11 are highly expressed in HeLa cells (Mostowy et al., 2009, Sellin et al., 2011), suggesting that there is no compensation for the depletion of SEPT9 (only SEPT3 group member expressed in HeLa cells). We cannot exclude that insufficient depletion of SEPT9 may also explain our findings that SEPT9 depletion does not significantly impact *S. aureus* invasion. Taken together, our data suggests that septins are involved in the signaling underlying actin rearrangements at sites of *S. aureus* invasion. Septins have been implicated in the invasion of other bacterial pathogens (such as *Listeria monocytogenes* and *Shigella flexneri,* whose entry does not rely on integrin α5β1), highlighting a fundamental role for septins in bacterial invasion (Aigal et al., 2022, Boddy et al., 2018, Mostowy et al., 2009, Mostowy and Cossart, 2012). There are important differences between septin interactions with invading bacteria (i.e. at the plasma membrane) versus cytosolic bacteria (i.e. septin cage entrapment of *S. flexneri*) as described in (Krokowski et al., 2018, Mostowy et al., 2009, Mostowy et al., 2010). Investigation of septin interactions with cytosolic *S. aureus* will be the focus of future work.

We discovered that protein levels of integrin α5, β1 and FAK are increased upon SEPT2 depletion. RT-qPCR indicated an increased expression of itga5 and itgb1 (albeit non-significantly different than control cells), suggesting that septins may coordinate the transcription of itga5 and itgb1. The actomyosin network has been described to modulate the activity of transcription regulators. Regulator such as YAP and TAZ are regulated by cell–

extracellular matrix adhesions and cytoskeletal tension (Halder et al., 2012). In addition, actomyosin has been suggested to work as a platform regulating ERK signalling in a tension-dependent manner (Hirata et al., 2015). Septins, via their association to actomyosin networks, may coordinate tension-dependent signalling which can impact the activity of transcriptional factors associated with FAs (Lam and Calvo, 2019). Recent work showed that SEPT2 and SEPT9 depletion caused a decrease in integrin β1 protein levels, highlighting that septins may have a context dependent role in FA biology (Kang et al., 2021). Future work is required to understand how septins may regulate integrin gene expression and/or protein stability.

We showed that SEPT2 depletion increased surface levels of integrin α5 and β1, as well as bacterial adhesion to the plasma membrane. These results are consistent with previous work showing that SEPT9 controls surface levels of epidermal growth factor receptors (Diesenberg et al., 2015), and that SEPT2 and SEPT11 control surface levels of Met receptor (Mostowy et al., 2011). The increase in surface levels of integrin α5 and β1 upon SEPT2 depletion may result from a trafficking defect, a slower rate of internalisation and/or an increased rate of recycling back to the plasma membrane. In support of this, a regulatory role for septins during endo-lysosomal sorting has previously been suggested (Song et al., 2016). In the future, it will be of great interest to test whether surface exposure of other membrane proteins involved in bacterial pathogenesis is affected by septin depletion.

Conclusion

Overall, our work highlights a fundamental role for septins in integrin α5β1 signaling. Future studies will be required to determine the precise contribution of septins during *S. aureus* invasion, and how these findings can be extended to other bacterial pathogens engaging integrin α5β1 for adhesion and cellular invasion (such as *Streptococcus pyogenes*) (Wang et

al., 2020). Considering that integrin α5β1 is also crucial for cell mechanotransduction (Lam and Calvo, 2019), it will be important to decipher the link between septins and signal transduction at FAs.

Material and methods

Reagents.

The following antibodies were used: rabbit anti-SEPT7 (#18991, IBL), mouse anti-active integrin α5β1 SNAKA51 (#MABT201, Sigma), rabbit anti-integrin α5 (#EPR7854, Abcam), mouse anti-integrin α5 conjugated with Alexa Fluor 488 (#53–0496–42, Invitrogen), mouse anti-integrin β1 (#14–0299–82, Thermofisher), mouse anti integrin β1 conjugated with Alexa Fluor 647 (#12G10, Abcam), rabbit anti-FAK (#12636–1-AP, ProteinTech), mouse anti-FAK (pY397) (#611806, BD Biosciences), mouse anti-GAPDH (#ab8245, Abcam), goat HRP-conjugated anti-mouse (#P0260, Dako), goat HRP-conjugated anti-rabbit (#P0448, Dako), Alexa-555-conjugated anti-rabbit antibody (#10082602, ThermoFisher), Alexa-647-conjugated anti-rabbit antibody (#A27040, ThermoFisher). The following dyes and drug were used: Hoechst (#H3570, ThermoFisher), Alexa-488-conjugated phalloidin (#A12379, ThermoFisher). Purified bovine plasma fibronectin (#F1141, Sigma) used for invasion assay was from SLS. The mCherry-Rab5 plasmid encoding mCherry-labeled human Rab5B protein was from Addgene (# 49201).

Bacterial strains and culture conditions.

S. aureus SH1000 producing cytosolic mCherry (mCherry-*S. aureus* SH1000) was used throughout the manuscript, expect for live cell imaging where a non-fluorescent *S. aureus*

RN6390 was used. *S. aureus* was grown in trypticase soy (TCS) agar (with 5 µg/ml erythromycin for mCherry-*S. aureus* SH1000). Clear conical bottom polypropylene tubes (#CLS430828, Corning) containing 5 ml of TCS were inoculated with individual colonies of *S. aureus* and were grown 16 h at 37 °C with shaking at 200 rpm. The following day, bacterial cultures were diluted in fresh prewarmed TCS (1:50 v/v), and cultured until an optical density (OD, measured at 600 nm) of 0.6.

Expression of GST-DuD4.

Expression and purification of GST-DuD4 was performed as described previously (Schröder et al., 2006). E. coli BL21 harboring pGEX-4 T-FnBPA-Du-D4 was grown at 37 °C, and protein synthesis induced with 1 mM isopropyl-β-d-thiogalactopyranoside. Purity of GST-DuD4 protein was analyzed by SDS-PAGE and Western blot by using goat anti-GST (diluted 1:1000; #G7781, Sigma). Protein concentrations were determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA).

Coating of proteins to latex beads.

Approximatively 1×10^9 latex beads (#LB11, Sigma) were washed and resuspended in 1 ml of sodium borate buffer (boric acid 1M, s96ydroxideroxyde 0.25M, pH 8.5) containing 0.5 mg/ml of GST-FnBPA-Du-D4. The protein was allowed to adsorb to beads overnight at 4°C. The solution was then incubated at room temperature (RT) for another 1 h. Beads were washed twice in borate sodium buffer then incubated for 1h with 5 mg/ml bovine serum albumin (BSA) in sodium borate buffer. After incubation, beads were washed and stored at 4°C in borate sodium buffer containing 5 mg/ml of BSA. For assays with cells, a beads-to-cell ratio of 10:1 was added to the cells.

Cell lines.

HeLa (ATCC CCL-2) cells were grown at 37°C and 5% CO2 in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Sigma). HeLa cells stably expressing GFP-SEPT6 and HeLa cells stably expressing GFP-SEPT6 and iRFP-LifeAct (Pfanzelter *et al.*, 2018) were grown as mentioned above in DMEM supplemented with 10% FBS and 2 μg/ml of puromycin.

Transfection and pharmacological inhibition.

HeLa cells (0.7×10^5) were plated in 6-well plates (Thermo Scientific) and transfected the following day. siRNA transfection was performed in DMEM with Oligofectamine (Invitrogen) according to the manufa'turer's instructions. Cells were tested 72 h after siRNA transfection. Control siRNA (#AM4611) and predesigned siRNA for SEPT2 (#14709), SEPT7 (#10323), SEPT9 (#18228) were all from Thermofisher (Sirianni *et al.*, 2016). Plasmid transfections were performed with JetPEI (Polyplus transfection) according to the protocol from the manufacturer. Cells were tested 24 h after transfection. For experiments involving pharmacological inhibitors, HeLa cells were treated for 30 min prior to infection and during infection with DMSO or Cytochalasin D (1 μM).

Infection of HeLa cells.

HeLa cells (1×10^5) were seeded in 12-well plates (Thermo Scientific) containing 18 mm glass coverslips 18 h before the infection. Bacterial cultures were grown as described and cell cultures were infected with *S. aureus* at MOI at various MOI. Cells were centrifuged at 110 g for 5 min at RT and plates were placed at 37 °C and 5% CO_2 for 15 min. Infected cultures were

washed 2 times with phosphate-buffered saline (PBS) pH 7.4 and either processed for microscopy analysis or gentamicin/lysostaphin protection assay.

For protection assays, gentamicin (100 µg/ml) lysostaphin (10 µg/ml) in DMEM was added to washed cells for 20 minutes to lyse all extracellular or adherent *S. aureus*. After incubation cells were washed 3 times in PBS and were lysed in H20 + 0.1% Triton X-100. Cell lysates were serially diluted and plated on TCS agar plates and incubated overnight at 37°C to determine the number of CFU.

Western blotting.

Samples were lysed in Laemmli buffer (Laemmli, 1970) and incubated at 95 °C for 10 min. Proteins were resolved in 10 SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PVDF, #IPVH00010, MerckMillipore). PVDF membranes were incubated with blocking solution for 1h at RT (75 mM Tris-HCl pH 8.8, 150 mM NaCl, 0.1% Tween20 supplemented with 5% milk). Incubation with the primary antibody (1 h 30 min) then with secondary goat HRP-conjugated antibodies (1 h) were performed in the blocking buffer at RT. PVDF membranes were washed 3 times x 5 min in blocking solution at RT and developed using PierceTM ECL plus western blotting substrate.

Immunostaining and fluorescence microscopy.

Infected or uninfected cells were washed 3 times with PBS pH 7.4 and fixed 15 min in 4% paraformaldehyde (in PBS) at RT. Fixed cells were washed 3 times with PBS pH 7.4 and subsequently permeabilized 5 min with 0.1% Triton X-100 (in PBS). Cells were washed 3 times in PBS and then incubated for 1h in blocking buffer (PBS supplemented with 0.1% Triton

X-100, 1% BSA and 10% guinea pig serum). After blocking, cells were incubated 1 h 30 min with primary anti-SEPT7 antibody diluted in blocking buffer. Cells were then washed 9 times in PBS and incubated 1h with Alexa fluor conjugated secondary antibody diluted in blocking buffer. Cells were then washed 9 times in PBS and coverslips were mounted with ProLong Gold antifade reagent with DAPI (#P36935, Thermofisher). Fluorescence microscopy was performed using a 63x/1.4 C-Plan Apo oil immersion lens on a Zeiss LSM 880 confocal microscope driven by ZEN Black software (v2.3) or Axiovert Z1 driven by ZEN Blue 2.3 software (Carl Zeiss). Microscopy images were obtained using z-stack image series and confocal images were processed using Airyscan processing with "Auto Filter" and "3D Processing" options. Image analysis was performed using Fiji. For live microscopy, cells were grown on MatTek glass-bottom dishes (MatTek corporation). For imaging, medium was changed to FluoroBrite™ DMEM+10% FBS and acquisition was performed using an Axiovert Z1 and temperature-controlled incubator (37°C).

Flow cytometry.

Hela cells were treated with siRNA as described above for 72 h. Cells were harvested in cooled centrifuge after trypsinisation and washed twice in 3 times with cold washing buffer (1xPBS and 5% FBS). After washing, cells were resuspended in 100 µl of the ice-cold wash buffer (containing 0.125 µg of anti-integrin α5 antibody conjugated with Alexa Fluor 488 and 0.1 µg of anti-integrin β1 antibody conjugated with Alexa Fluor 647) and incubated at 4°C in the dark for 45 minutes. Cells were then washed 3 times with cold washing buffer and the individual cells were measured by using flow cytometry with an LSRII (BD Biosciences) or Attune™ NxT (Thermo Fisher Scientific). The data were analysed using FlowJo software, version 10.7.1. The mean fluorescence of the cell population was determined and plotted for each staining (see figure legend).

RT-qPCR.

RNA was extracted with a RNeasy minikit (Qiagen) and reverse-transcribed using a QuantiTect reverse transcription kit (Qiagen) according to the the manufacturer's instructions. Template cDNA was subjected to PCR using primers for gene encoding for integrin a5 (#HP205940, Origene), integrin b1 (#HP232479, Origene) and FAK (#HP208715, Origene). Quantitative reverse transcription PCR (RT-qPCR) was performed using 7500 Fast Real-Time PCR System machine and 7500 Fast Real-Time PCR software v2.3 (Applied Biosystems, Foster City, California) and SYBR green master mix (#10187094, Applied Biosystems). Primers against housekeeping gene GAPDH (Sirianni *et al.*, 2016) and the delta-delta-Ct method were used for gene expression quantification.

Quantification and Statistical Analysis.

Statistical analysis was performed in Excel (Microsoft) and GraphPad Prism 9.4.1 (La Jolla, USA). Unless otherwise indicated, data represent the mean ± standard error of the mean (SEM) from at least 3 independent experiments. A Student's t-test (two-tailed) or one-way ANOVA was used to test for statistical significance, with $p < 0.05$ considered as significant. All details on statistical analysis (including statistical tests, significance, value of the number of experimental replicates, and bacterial cells quantified) can be found in the figure legends.

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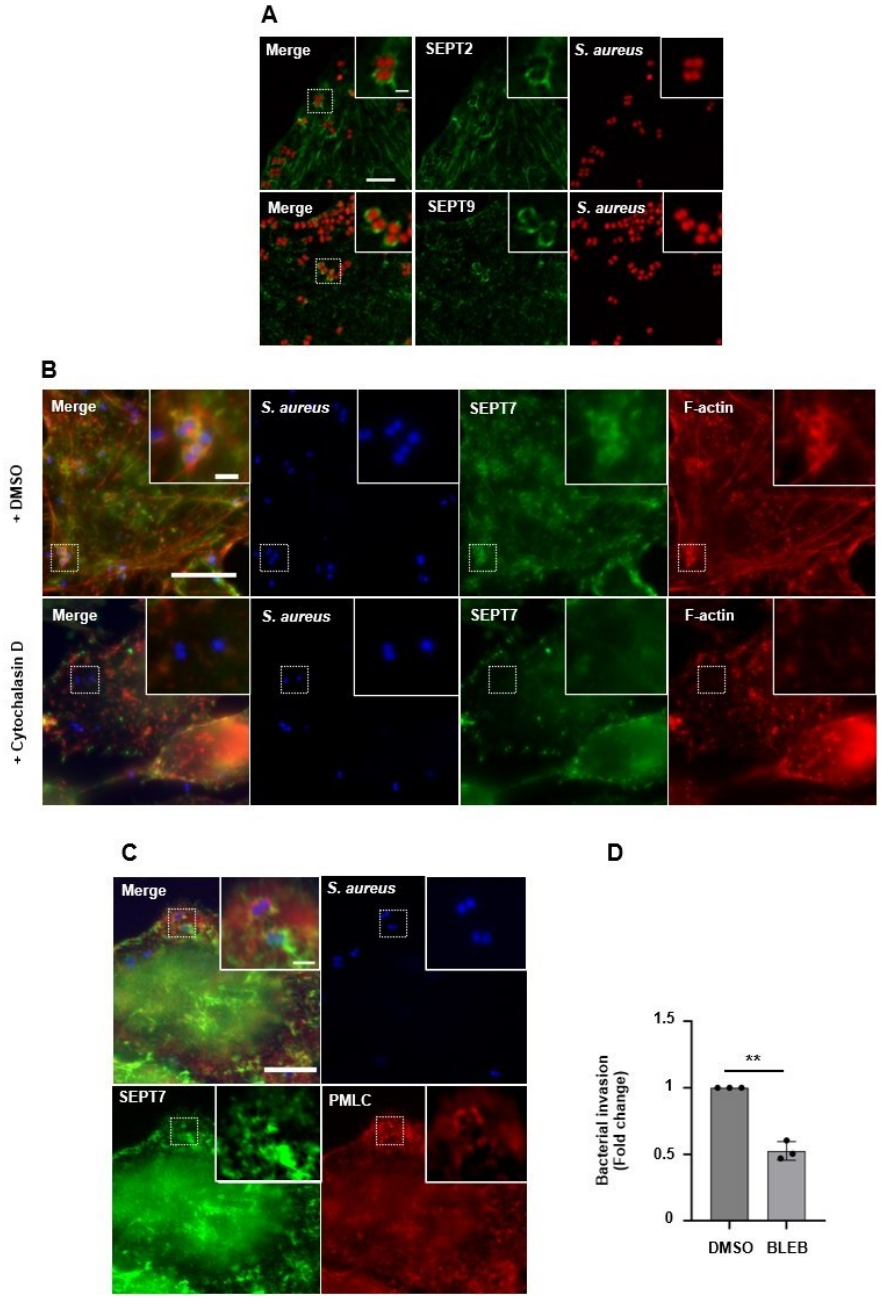
Author Contributions.

SR and SM conceived the study and wrote the manuscript and all other authors commented on the manuscript. SR, DB, DLM designed experiments and analyzed results.

Declaration of Interests.

The authors declare that they have no conflict of interest.

Supplemental Figure 1.



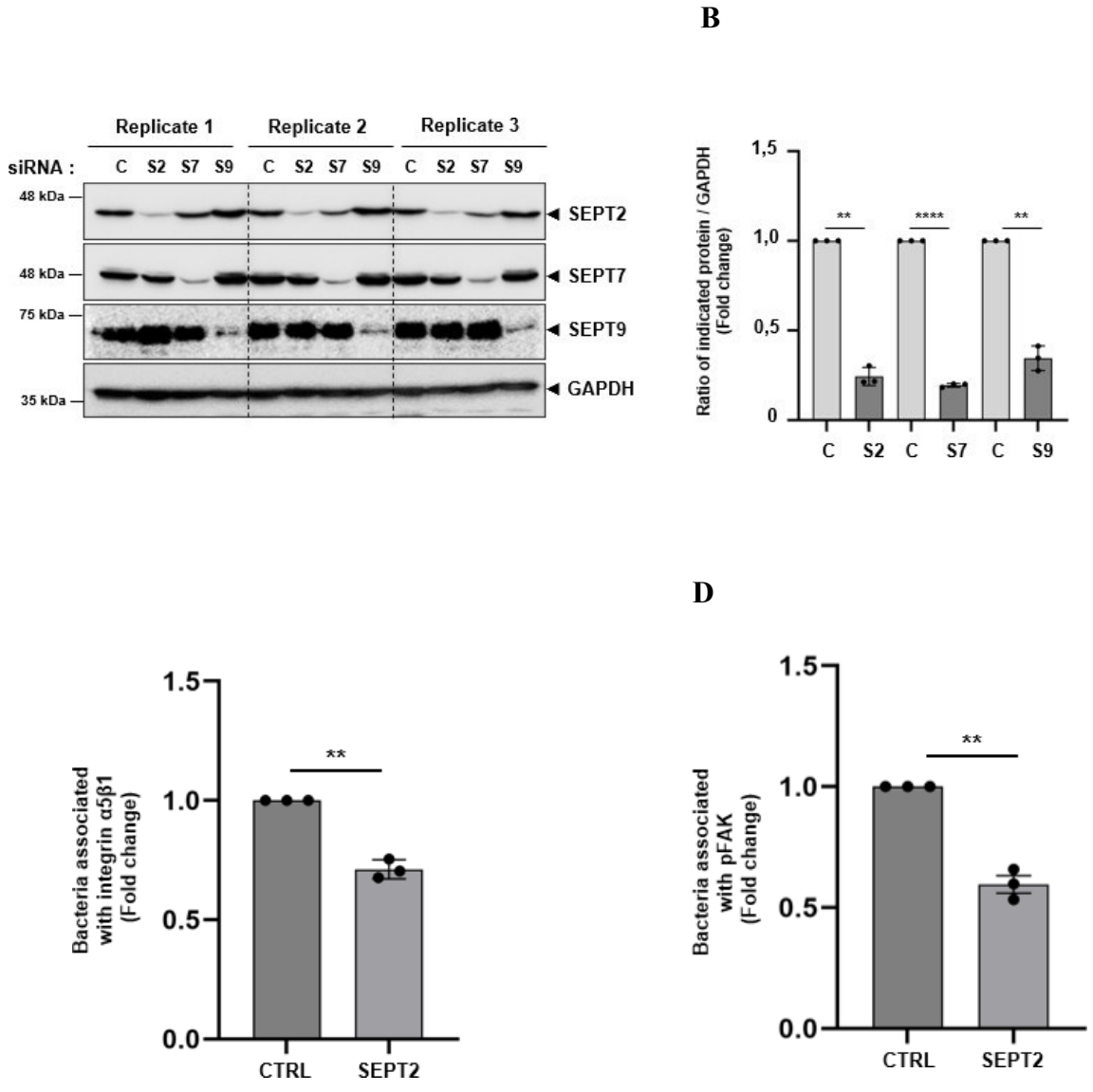
(A) HeLa cells were infected with mCherry-*S. aureus* with a MOI of 20 in DMEM+10% FBS and fixed 15 min post-infection. Cells were stained for SEPT2 (top row) or SEPT9 (bottom row). Scale bars represent 10 µm (main image) and 2 µm (inset).

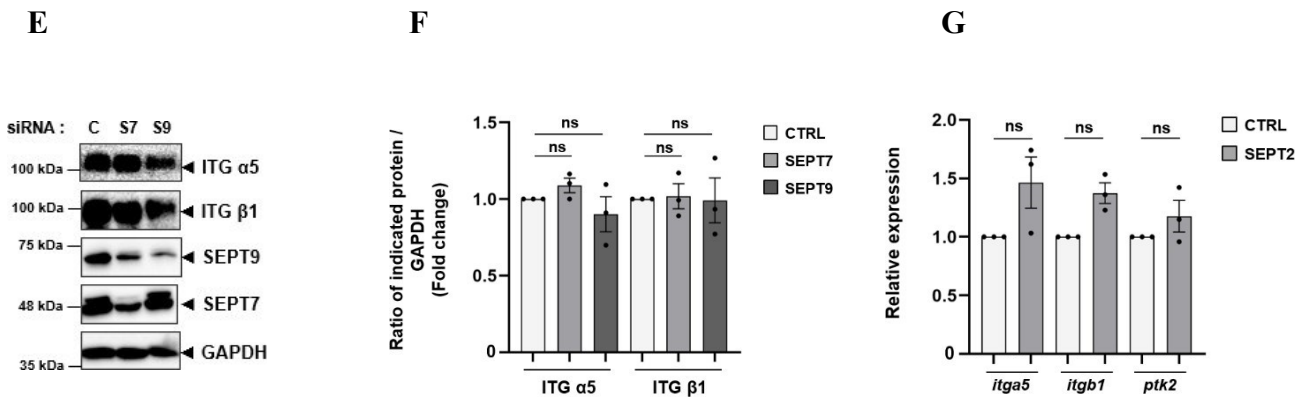
(B). HeLa cells were treated with DMSO as control (top row) and 1µM Cytochalasin D (bottom row) for 40 min and infected with mCherry-*S. aureus* for 15 min. After fixation, cells were stained for SEPT7 (green) and F-actin (red). Scale bars represent 15 µm (main image) and 2 µm (inset). Loss of F-actin can be observed upon cytochalasin D treatIt.

(C) HeLa cells were infected with mCherry-*S. aureus* with a MOI of 20 in DMEM+10% FBS and fixed 15 min post invasion. Cells were stained for SEPT7 and phosphorylated myosin light chain IIA (PMLC). Scale bars represent 10 µm (main image) and 2 µm (inset).

(D) HeLa cells were treated with DMSO as control and 50 µM of blebbistatin for 30 min and infected with mCherry-*S. aureus* for 30 min. Gentamicin-lysostaphin protection assays were performed and infected cells were lysed and plated on TCS agar plates. The graph represents the CFU normalized to the DMSO treated samples. The CFU ± SEM from 3 independent experiments is presented. **, $p < 0.01$ by two-taile' Student's t-test

Supplemental Figure 2.





(A) HeLa cells were transfected with the indicated siRNA for 72 h (C : control; S2 : SEPT2; S7 : SEPT7; S9 : SEPT9). Post-siRNA transfection, cells were lysed and whole-cell lysates were immunoblotted for SEPT2, SEPT7 or SEPT9 to show the efficiency of depletion. GAPDH was used as a loading control. Western blot for 3 independent experiments is shown.
(B) Densitometry-based quantification of SEPT2 (S2), SEPT7 (S7) and SEPT9 (S9) signal from (A). All bands were first normalized to the signal of the loading control GAPDH. Graph represents the fold change of the indicated protein normalised to control siRNA treated cells ± SEM from 3 independent experiments. **, $p < 0.002$, *****, $p < 0.0001$ by two-taile' Student's t-test.
(C) HeLa cells were transfected with the indicated siRNAs for 72 h. Post-siRNA transfection, cells were infected with *S. aureus* for 15 min in DMEM + 10% FBS. After thorough washing steps (no gentamicin-lysostaphin incubation step) cells were fixed and stained for SEPT7 and integrin α5β1. The graph represents mean percentage ± SEM normalized to cells treated with control siRNA and SEM of bacteria associated with integrin α5β1 from n = 1243 (CTRL siRNA) or n = 1272 (SEPT2 siRNA) bacteria in three independent experiments. **, $p < 0.001$ by two-taile' Student's t-test.
(D) HeLa cells were transfected with the indicated siRNAs for 72 h. Post-siRNA transfection, cells were infected with *S. aureus* for 15 min in DMEM + 10% FBS. After thorough washing steps (no gentamicin-lysostaphin incubation step) cells were fixed and stained for SEPT7 and phosphorylated FAK (pFAK). The graph represents mean percentage ± SEM normalized to cells treated with CTRL siRNA and SEM of bacteria associated with integrin α5β1 from n = 903 (CTRL siRNA) or n = 1346 (SEPT2 siRNA) bacteria in three independent experiments. **, $p < 0.001$ by two-taile' Student'sItest.
(E) Western blot using whole-cell lysates from control siRNA or SEPT2 siRNA treated HeLa cells. Integrin α5 (ITG α5), integrin β1 (ITG β1), SEPT7 and SEPT9 (to show depletion efficiency) protein levels were detected. GAPDH protein level was used as a loading control.
(F) Densitometry-based quantification of integrin α5 (ITG α5) and integrin β1 (ITG β1) Inal from (E). All bands were first normalized to the signal of the loading control GAPDH. Graph represents the fold change of the indicated protein normalised to control siRNA treated cells (CTRL) ± SEM from 3 independent experiments. ns: non-significant by two'tailed Student's t-test.
(G) HeLa cells were transfected with the indicated siRNAs for 72 h. Post-siRNA transfection, RNA was extracted, and cDNA were prepared to perform RT-qPCR. Graph represents fold change in the expression of gene encoding for integrin α5 (*itga5*), integrin β1 (*itgb1*) and FAK (*ptk2*) normalised to control siRNA treated cells (CTRL) ± SEM from 3 independent experiments. ns: non-significant by two tailed Student's t-test.

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3.1.2.4. Conclusion

In this chapter we described for the first time a role for septins in *S. aureus* invasion, where septins are recruited with actin to *S. aureus* invasion sites engaging integrin α5β1. We showed that septin depletion reduced *S. aureus* invasion, but increased surface expression of integrin α5 and adhesion of *S. aureus* to host cells. Septin depletion led to increased cellular protein levels of integrin α5 and β1 subunits.

Together, these results highlight a novel role for septins in regulation of integrin α5β1 and invasion of *S. aureus*. A recent study suggested that, similar to *S. flexneri* and *P. aeruginosa*, septins were recruited to intracellular *S. aureus* during infection of HeLa cells (Krokowski *et al.*, 2018). In this following chapter we set out to investigate this preliminary observation.

Chapter 4. Investigating intracellular *S. aureus* interactions with the septin cytoskeleton

4.1. Introduction

Many intracellular bacteria interact with the host cytoskeleton to ensure their survival and dissemination. Examples include *L. monocytogenes* and *S. flexneri* actin-based motility, or the *C. trachomatis* actin/septin coat to promote extrusion from infected cells (Torraca and Mostowy, 2016). The study of bacterial strategies used to modulate the host cytoskeleton has significantly contributed to our understanding of function and regulation of cytoskeletal pathways (Haglund and Welch, 2011).

Generally, upon invasion of NPPCs, intracellular *S. aureus* initially reside in phagolysosomes, a degradative bactericidal compartment. *S. aureus* possess an arsenal of pore-forming toxins to escape this compartment and reach the cytosol where it replicates and causes host cell death (Moldovan and Fraunholz, 2019).

The ability of *S. aureus* to transition from colonization to invasion, vacuolar localization to cytosolic escape requires extensive alteration of bacterial gene expression to adapt to the new micro-environment and potentially respond to cell-autonomous immune mechanisms (Howden *et al.*, 2023). A microarray analysis using infection of human lung epithelial A549 cells showed an alteration of gene expression related to virulence, metabolism, nutrient acquisition and CW synthesis (Garzoni *et al.*, 2007). Comparative transcriptomic studies of *S. aureus* across different experimental conditions (including growth in different media at different growth stages, as well as to internalization by different eukaryotic host cells) provided an important link between culture condition, growth phase and infection outcomes (Malachowa *et al.*, 2011;

Mäder *et al.*, 2016). Throughout bacterial growth, factors such as cell density, pH of the growth medium and nutrient availability are changing. A complex regulatory network including the Agr quorum system, the cytoplasmic SarA-family regulators (SarA, Rot and MgrA), the alternative sigma factors (SigB and SigH) and the two-component system SaeRS were linked to rapid adaptation of *S. aureus* to environmental changes by coordinating the expression of a large number of secreted toxins and cell-surface components (Jenul and Horswill, 2019).

Ihe adoption of an intracellular lifestyle by *S. aureus* is increasingly recognized as a critical aspect of its pathogenesis (Soe *et al.*, 2021). This privileged intracellular niche offers a window of opportunity to evade host immune cells and antibiotics. The complex nature of host–pathogen interactions during *S. aureus* infection is illustrated by the fact that *S. aureus* post-invasion events are highly influenced by both the host cell type and the infecting strain (Strobel *et al.*, 2016). *S. aureus* infection outcomes were shown to be highly dependent on MOI, potentially because the confined phagosome heightened rapid activation of the Agr system (Pang *et al.*, 2010). On the host side, changes in the host cell transcriptional profile of infected endothelial cells have also been described, with alterations of various cellular signaling pathways related to immunity such as interferon signaling, MHC Class I presentation, and phagocytosis (Matussek *et al.*, 2005; Grønnemose *et al.*, 2021).

Inside cells, *S. aureus* is exposed to cell-autonomous immune processes such as autophagy, but whether autophagy is beneficial or detrimental to *S. aureus* is controversial. In fibroblasts, *S. aureus* may use autophagosomes as a niche for bacterial replication and inhibition of autophagy led to the reduction of intracellular bacteria. Similarly, inhibition of autophagy by overexpression of protein kinase C restricted *S. aureus* intracellular replication in Chinese hamster ovary cells (Gauron *et al.*, 2021). In HeLa cells, *S. aureus* was observed to be replicating in non-acidified autophagosomes and pharmacological activation of autophagy led to a higher intracellular bacterial load. In contrast, the knockout of *atg5*, a key gene required

for autophagy, from murine fibroblasts NIH/3T3 led to increased intracellular replication (Neumann *et al.*, 2016). In a zebrafish infection model, autophagy can be protective or detrimental to the host. The autophagy marker microtubule-associated protein 1A/1B-light chain 3 (LC3) rapidly decorates *S. aureus* following phagocytosis by macrophages and neutrophils to provide an intracellular niche for bacterial pathogenesis. However, intracellular bacteria can also be targeted by SQSTM1 which is host-protective in this case (Prajsnar *et al.*, 2021).

Another emerging cell-autonomous immune mechanism is mediated by guanylate-binding proteins (GBPs) which have been reported to target the LPS of Gram- pathogens such as *S. flexneri*, leading to bacterial membrane rupture and activation of host inflammasome. However no direct binding of GBPs could be observed in the case of Gram+ pathogens such as *L. monocytogenes* or *S. aureus* highlighting the specificity of this defense pathway for some bacterial factors (Kutsch *et al.*, 2020; Feng *et al.*, 2022). Therefore, a better characterization of host–*S. aureus* interactions during intracellular infection is required to gain new insights into how host cell pathways can be modulated against this versatile facultative intracellular pathogen.

Considering the emerging roles of the septin cytoskeleton in host-pathogen interactions (as observed using a variety of bacterial pathogens) (Robertin and Mostowy, 2020), along with initial work suggesting an association between septins and intracellular *S. aureus* (Krokowski *et al.*, 2018), we believed that characterisation of *S. aureus*-septin caging represented an important avenue to investigate.

4.2. Results

4.2.1. Use of HeLa YFP-CWT to study septin interactions with intracellular *S. aureus*

In a recent paper from the lab using HeLa cells overexpressing RFP-SEPT6, it was shown that septins could be observed at the division site of intracellular *P. aeruginosa* and *S. aureus,* and the recruitment of endogenous septins to intracellular *P. aeruginosa* was confirmed using WT HeLa cells (PhD dissertation of S. Krokowski, 2019, unpublished results). In the case of *S. aureus*, no further investigations have been done beyond that preliminary observation. This motivated us to follow-up on this observation, and to investigate whether septins can potentially entrap *S. aureus* in cage-like structures, similarly to *S. flexneri* and *P. aeruginosa.*

To test this we used HeLa cells transiently expressing a cytosolic "*S. aureus* escape reporter" (Strobel *et al.*, 2016). This escape reporter is a yellow fluorescent tagged CW targeting domain (YFP-CWT), which shows strong affinity for *S. aureus* CW. Upon rupture of *S. aureus* lysosomal compartment, YFP-CWT rapidly decorates the bacterial cell wall, allowing to distinguish vacuolar versus cytosolic bacteria. Cells were infected with *S. aureus* SH1000-mCherry with an MOI of 20 for 30 min and treated with lysostaphin to lyse all extracellular bacteria. The interaction of septin with vacuolar vs cytosolic bacteria were investigated at 1h post-treatment by widefield epifluorescence microscopy.

Although we could observe local septin enrichment at the same XY location of intracellular bacteria, this enrichment appeared to be at a different Z location as compared to the bacteria. This can lead to false positive events of "septin-caging" of intracellular *S. aureus*. To exclude possible fluorescence artifacts due to Z-stack resolution limitation of epifluorescence microscopy, we used high resolution Airyscan confocal microscopy. Reconstructed XZ-

confocal cross-section images clearly showed that septins were not accumulating around intracellular bacteria as would be expected for a septin cage. Instead septins were observed apically of the bacteria as shown by the Z-stacks, with septin enrichment becoming apparent only when moving towards the cell surface (**Figure 10 A and B**). Considering the curvature-sensing ability of septins, this local enrichment is possibly happening because septins sense the plasma membrane deformation generated to accommodate the intracellular bacteria (Bridges *et al.*, 2016; Lobato-Márquez *et al.*, 2021).

Despite intense investigation, virtually no septin enrichment around intracellular *S. aureus* reminiscent of the *S. flexneri* and *P. aeruginosa* septin cage were detected, except for 1 single event of a cell displaying clear septin accumulation following the contour of cytosolic bacteria at each Z slices (**Figure 11 A and B**). This was the only cell harbouring septin-positive bacteria in 3 biological replicates (150 cells quantified in total). In this case, reconstructed XZ-confocal cross-section images clearly showed that cytosolic *S. aureus* were fully entrapped by septins as would be expected for a septin cage, in contrast to what was observed in **Figure 10 B**. Although this could be an artifact due to YFP-CWT overexpression affecting *S. aureus* surface properties and/or septin function, this result could also suggest that septins may associate with cytosolic *S. aureus* but our experimental conditions might not be optimal to reliably observe these events.

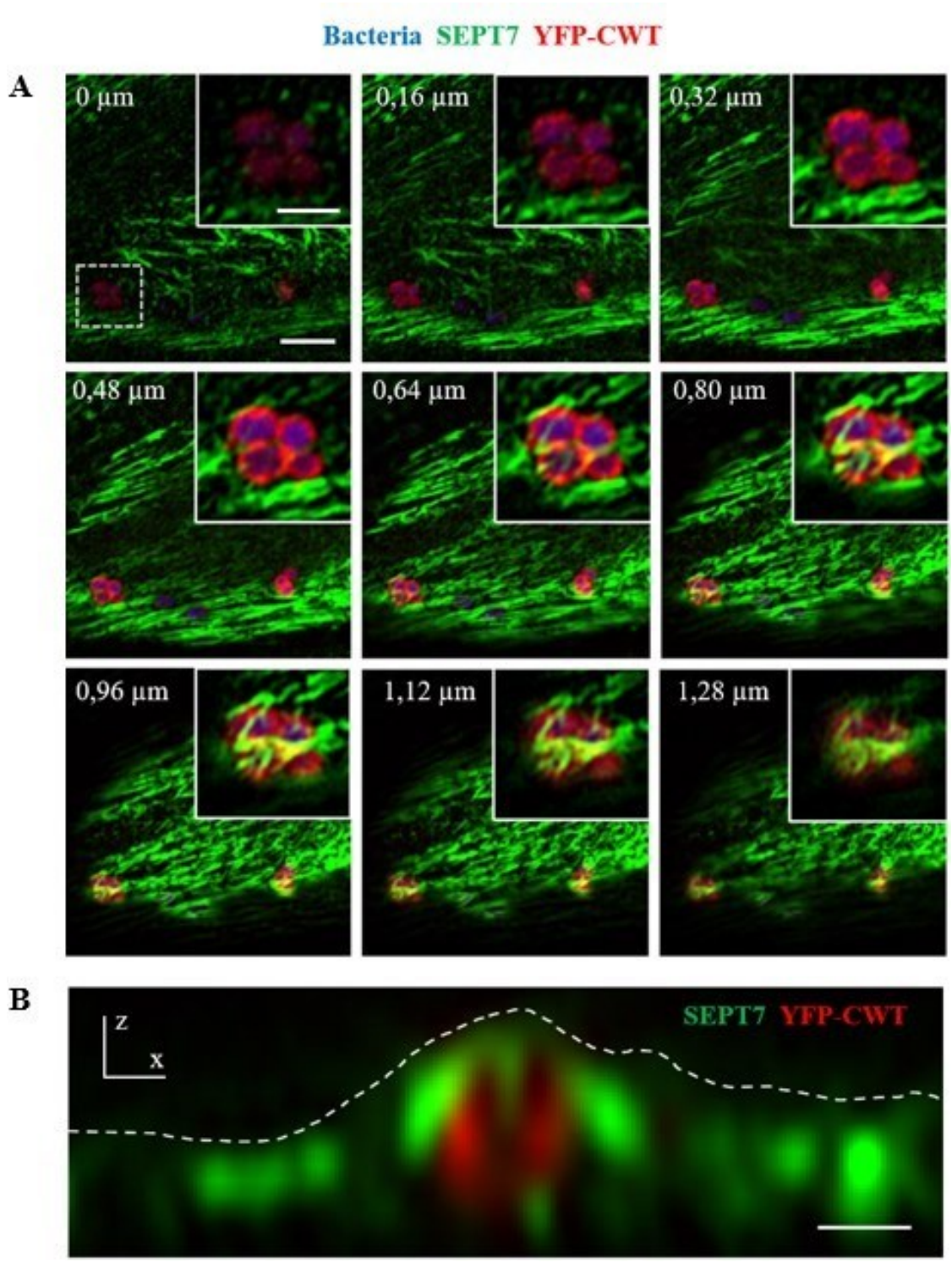


Figure 10. Local septins enrichment apically of intracellular *S. aureus*. A. Airyscan confocal z-stack images with a stepsize of 0,16 µm between each captured optical sections. HeLa cells transiently expressing the *S. aureus* escape marker YFP-CWT (shown in red here) were infected with *S. aureus* SH1000-mCherry (shown in blue) at MOI of 20 in DMEM + 10% FBS for 30 min, lysostaphin-treated and fixed 1h00 post-treatment. Cells were stained for SEPT7 (green). Scale bars represent 15 µm(main image) and 2 µm (inset). **B.** Reconstructed XZ-confocal cross-section images data of the intracellular bacteria from inset in (A). Scale bar represents 1 µm. SEPT7 (green) and escape marker (red) are shown. Dashed outline is shown to represent cell contour.

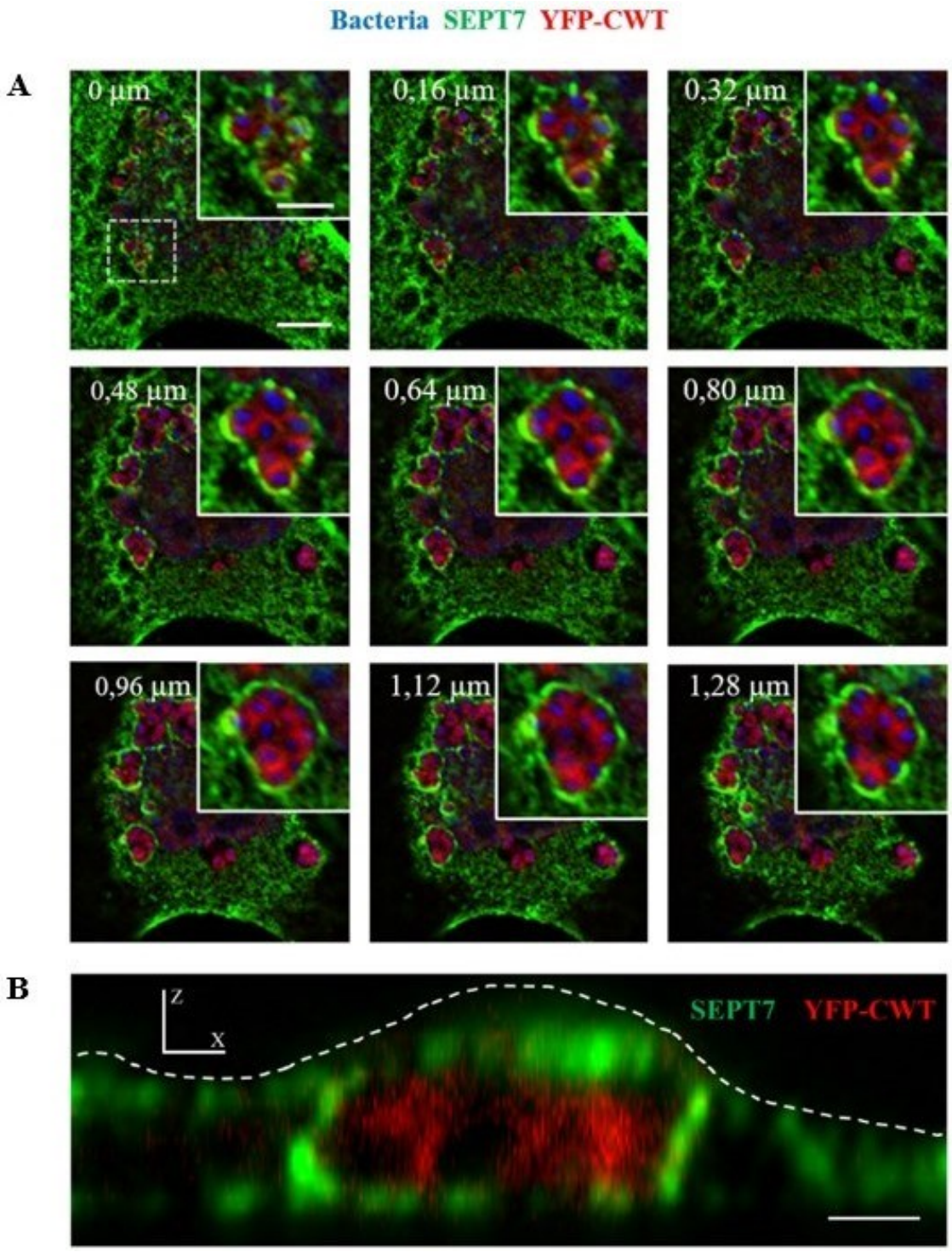


Figure 11. Septin caging of intracellular *S. aureus*. A. Airyscan confocal z-stack images with a stepsize of 0,16 µm between each captured optical sections. HeLa cells transiently expressing the *S. aureus* escape marker YFP-CWT (shown in red here) were infected with *S. aureus* SH1000-mCherry (shown in blue) at MOI of 20 in DMEM + 10% FBS for 30 min, lysostaphin-treated and fixed 1h00 post-treatment. Cells were stained for SEPT7 (green). Scale bars represent 15 µm (main image) and 2 µm (inset). **B.** Reconstructed XZ-confocal cross-section images data of the intracellular bacteria from inset in (A). Scale bar represents 1 µm. SEPT7 (green) and escape marker (red) are shown. Dashed outline is shown to represent cell contour.

4.2.2. Use of WT HeLa cells to study septin interactions with intracellular *S. aureus*

Considering that the overexpressed YFP-CWT construct has a strong affinity for *S. aureus* CW, its binding might interfere with potential septin interactions. Thus, we performed experiments using non transfected HeLa cells infected with *S. aureus* SH1000-mCherry for 1h and 3h post-lysostaphin treatment to investigate early and late events in the infection process, respectively. We were not able to investigate later time-points as cytosolic escape and active intracellular replication of *S. aureus* triggered host cell death (Strobel *et al.*, 2016). Cells were immunostained for SEPT7 and LAMP1 (lysosomal marker). Similar to what was observed using HeLa cells overexpressing YFP-CWT, we were not able to reliably observe septin cage entrapment of intracellular *S. aureus* (**Figure 12**).

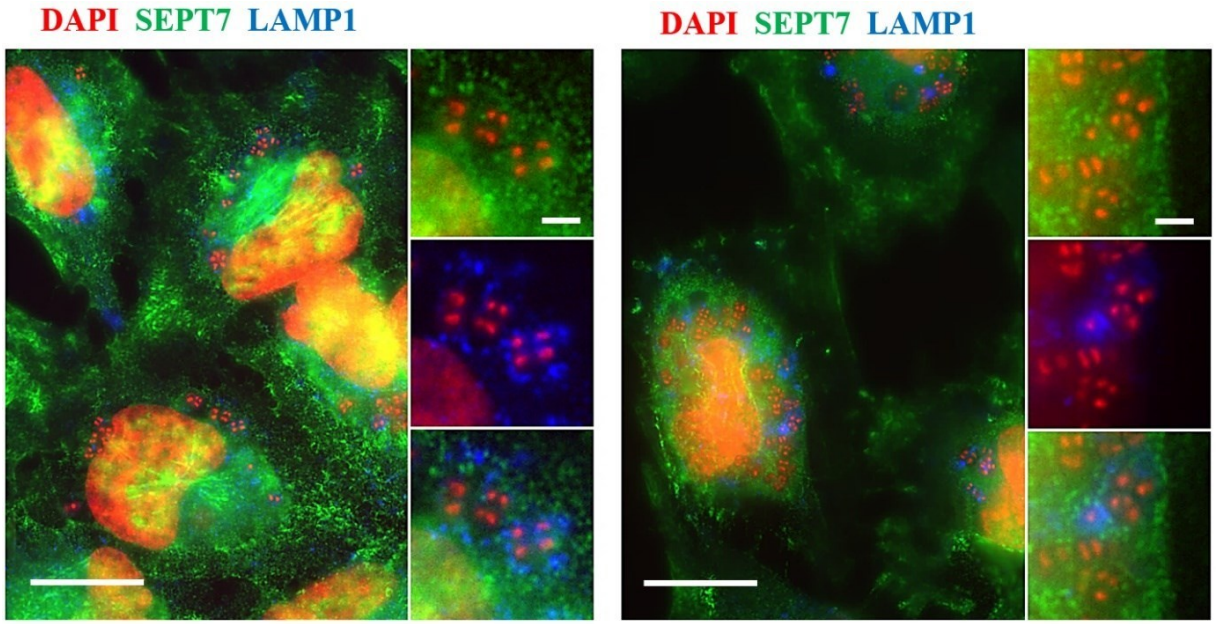


Figure 12. Incubation period does not affect septins-*S. aureus* interactions. HeLa cells were infected with *S. aureus* SH1000-mCherry at MOI of 20 in DMEM + 10% FBS for 30 min, lysostaphin-treated and fixed 1h post-treatment (left) or 2h30 post-treatment (right). Cells were stained for SEPT7 (green), LAMP1 (blue) and DAPI to label DNA (red). Scale bars represent 15 µm (main image) and 2 µm (inset).

4.2.3. Investigating septin-*S. aureus* interactions under various experimental conditions

To gain further insights into potential septin-*S.aureus* interactions, we conducted a series of experiments under diverse experimental conditions which could influence both bacteria and host-cell physiology, ultimately impacting host-pathogen interactions (for each conditions tested, biological triplicates were performed and > 100 cells were quantified). Unfortunately, none of these experimental conditions tested resulted in clear septin association with intracellular *S. aureus*. Below we summarize these experimental conditions :

4.2.3.1. Infection at lower (1) and higher (100) MOI

Considering how the intracellular bacterial load can affect both bacterial (e.g. Agr quorum sensing response) and host physiology (e.g. change in host cell signalling and transcriptional response) we investigated whether MOI can affect septin recruitment to *S. aureus* SH1000-mCherry (**Figure 13**). However, MOI did not influence septin-caging of *S. aureus*.

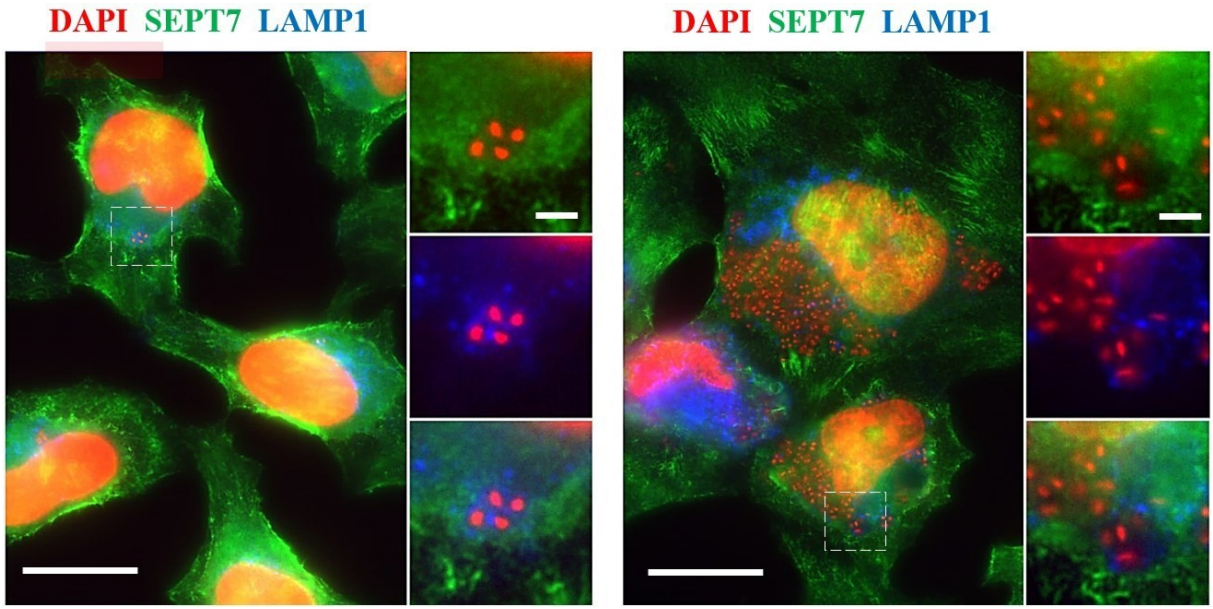


Figure 13. MOI does not affect septins-*S. aureus* interactions. HeLa cells were infected with *S. aureus* SH1000-mCherry at MOI of 1 (**A**) or 100 (**B**) in DMEM + 10% FBS for 30 min, lysostaphin-treated and fixed 2h30 post-treatment (**B).** Cells were stained for SEPT7 (green), LAMP1 (blue) and DAPI to label DNA (red). Scale bars represent 15 µm (main image) and 2 µm (inset).

4.2.3.2. Infection using bacteria with different metabolic profiles

Similar to MOI, bacterial growth stage and growth medium composition can substantially affect bacterial transcriptomic profiles, virulence and infection outcomes. To test these parameters, we infected HeLa cells with *S. aureus* SH1000-mCherry collected at early (OD_{600} ~ 2) and late stationary growth phase (overnight culture, 18h) in TSB (**Figure 14**). Of note we also infected HeLa cells with bacteria grown (overnight and exponentially) in cell culture medium (DMEM + 10% FBS). These parameters did not affect septin-caging of *S. aureus*.

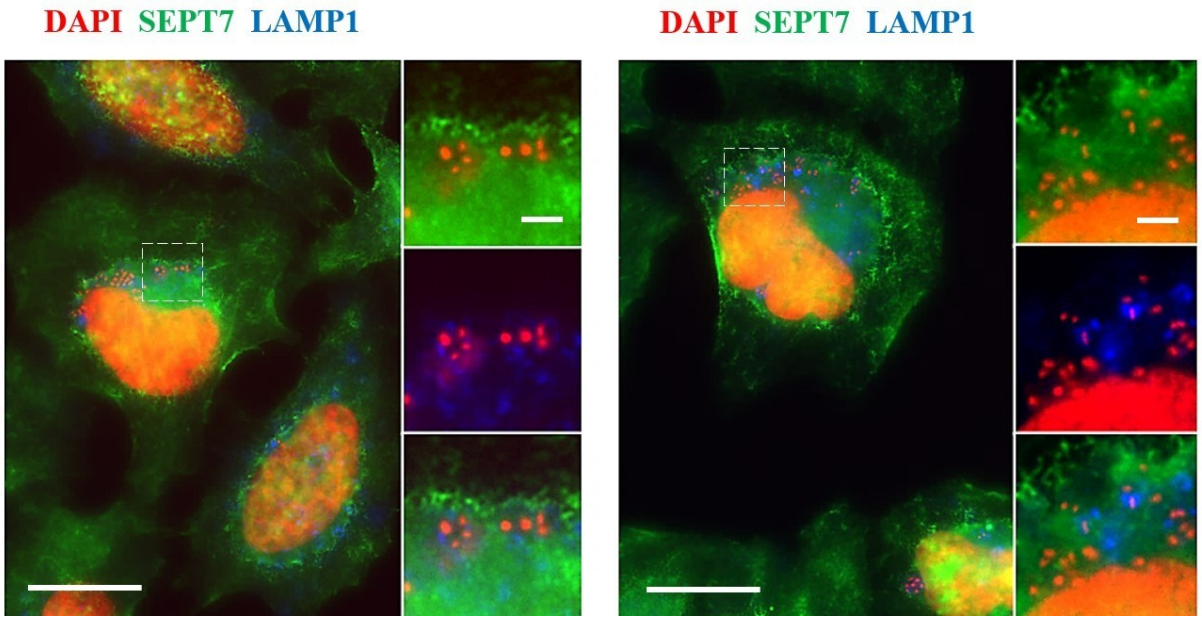


Figure 14. Bacterial metabolic activity does not affect septins-*S. aureus* interactions. HeLa cells were infected at MOI 20 in DMEM + 10% FBS for 30 min with either *S. aureus* SH1000-mCherry collected at early (left) or late stationary growth stage (right). Cell were fixed 2h30 post-lysostaphin treatment**.** Cells were stained for SEPT7 (green), LAMP1 (blue) and DAPI to label DNA (red). Scale bars represent 15 µm (main image) and 2 µm (inset).

4.2.3.3. Infection of human broncho-epithelial and fibroblast cell lines

Post-invasion events during *S. aureus* infections are highly dependent on host cell type, as cell-type specific regulation of cellular pathway can influence host response. We investigated septin interactions with intracellular *S. aureus* SH1000-mCherry in two other NPPC, human

fibroblast (SV40-transformed) and human broncho-epithelial (16HBE14o-) cell lines. Again, we were not able to identify septin-caging of intracellular *S. aureus* (**Figure 15**).

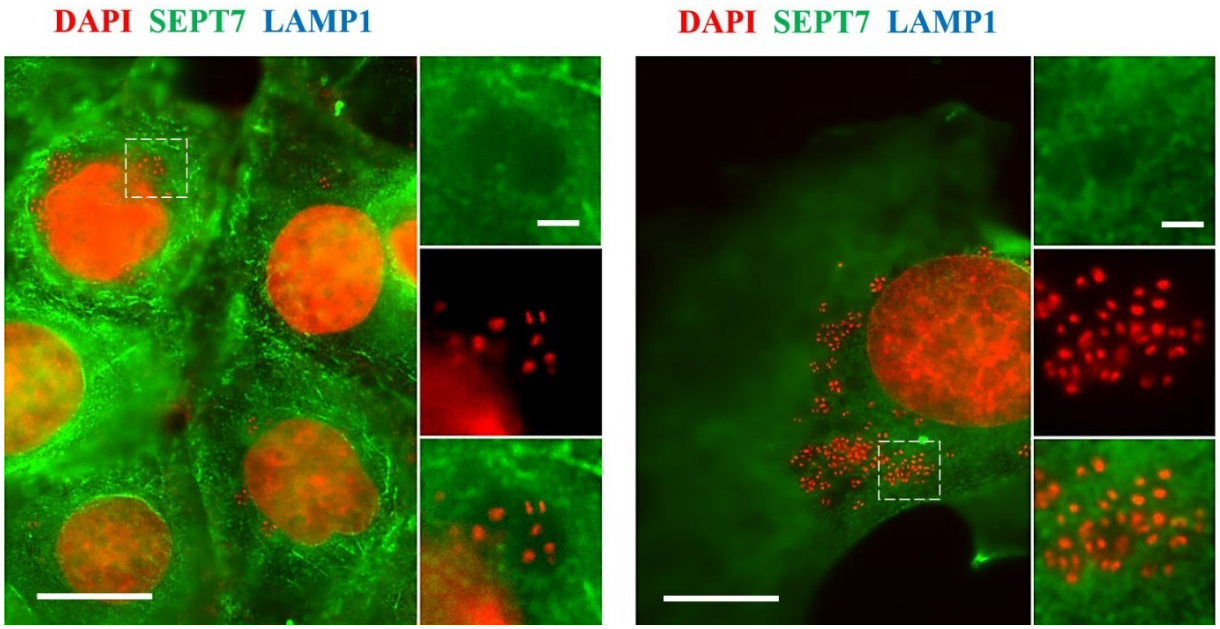


Figure 15. Cell type does not affect septins-*S.aureus* interactions. Human broncho-epithelial cells 16HBE14o- (**A**) and sv40-transformed human fibroblasts (**B**) were infected with *S. aureus* SH1000 mCherry at MOI of 20 in DMEM + 10% FBS for 30 min, lysostaphin treated and fixed 2h30 post-treatment. Cells were stained for SEPT7 (green) and DAPI to label DNA (red). Scale bars represent 15 µm (main image).

4.2.3.4. Infection with different *S. aureus* strains

Post-invasion events are highly dependent on the selection of infecting *S. aureus* strains which can present different levels of cytotoxicity and expression of virulence factors. We assessed whether we could observe septin association to different *S. aureus* strains, the MRSA COL and a virulent MSSA clinical isolate expressing the toxin Panton-Valentine Leukocidin (MSSA PVL+) (**Figure 16**). Our results showed that the choice of strain does not affect septin-caging of *S. aureus*.

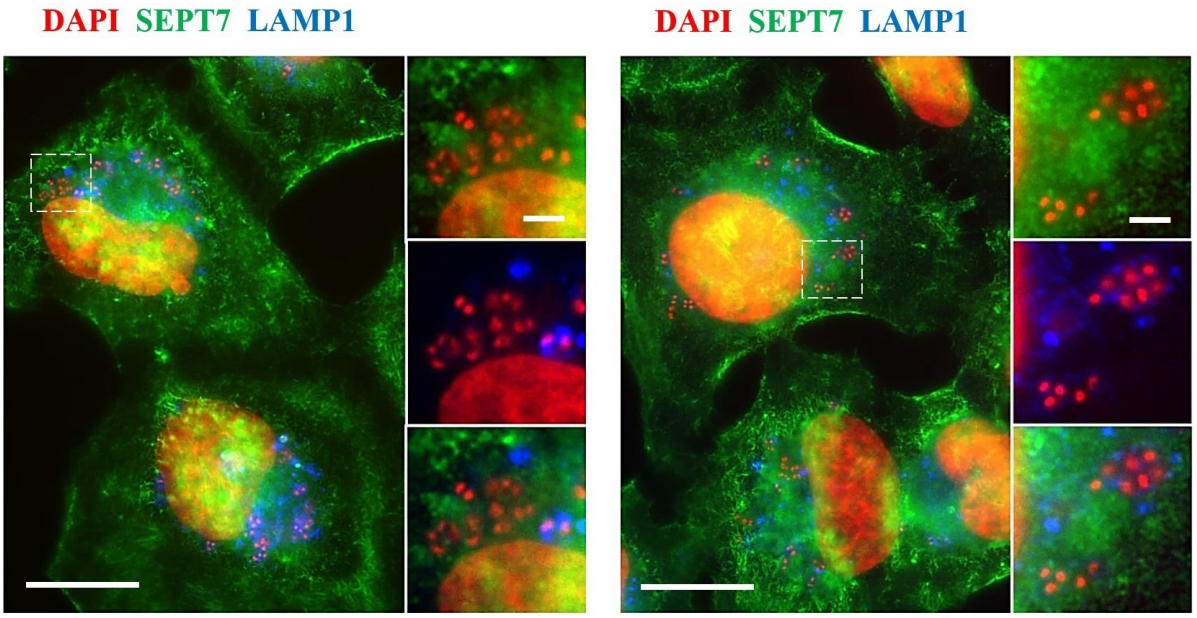


Figure 16. Bacterial strain does not affect septins-*S.aureus* interactions. HeLa cells were infected with MRSA COL (**A**) or MSSA PVL+ (**B**) at MOI of 20 in DMEM + 10% FBS for 30 min, lysostaphin treated and fixed 2h30 post-treatment. Cells were stained for SEPT7 (green), LAMP1 (blue) and DAPI to label DNA (red). Scale bars represent 15 µm (main image).

Taken together, we conclude that septins do not entrap intracellular *S. aureus* in the different experimental conditions tested here. Our results suggested that the phenotype of septin-caging observed in **Figure 11** might be an artifact due to YFP-CWT overexpression, or alternatively, *S. aureus* secreted or surface factors might be actively preventing septin binding, similarly to what was suggested for the Gram+ intracellular pathogen *L. monocytogenes* (Mostowy *et al.*, 2010).

4.2.4. Discussion

The septin cytoskeleton has emerged as a key player in host-pathogen interactions, as revealed by infection from of a large variety of Gram+ and Gram- bacterial pathogens (Robertin and Mostowy, 2020). In the case of *S. aureus* infection, an involvement of the septin cytoskeleton had not been studied. In **Chapter 3** we characterised a functional role for septins

in integrin α5β1-mediated cellular invasion of *S. aureus*. Our lab previously observed septin association with intracellular *S. aureus*, so in **Chapter 4** we further investigated this interaction.

In a first step we wanted to distinguish septin recruitment to vacuolar vs cytosolic bacteria using HeLa cells overexpressing a *S. aureus* escape marker. However, septin-*S. aureus* interactions were not reliably observed. We performed various experiments in which we modulated different experimental parameters, ranging from diverse MOIs to use of different bacterial strains and cell lines, to assess whether this will lead to a more clear and more robust septin caging phenotype. Despite our extensive efforts, we failed to reliably observe septin caging of intracellular *S. aureus* in any of the experimental conditions we tested here. At this stage there could be different explanations.

Septin interactions with *S. aureus* may also be transient and dynamic, and thus difficult to capture and quantify, which is further complicated by the high background of other septin structures present in the cytosol. To address this challenge, high-resolution live microscopy techniques would be necessary to track and study these interactions in real-time.

Alternatively, it is possible that *S. aureus*, through secreted or surface factors, and/or host factors co-opted by the bacteria, can actively preventing septin binding, a scenario similarly to that suggested for *L. monocytogenes* (Mostowy *et al.*, 2010).

The specificity of host defense mechanism, such as septin caging towards Gram- bacteria, would not be a surprising concept, as for example GBPs have been shown to target specifically Gram- bacteria and not Gram+ (such as *L. monocytogenes* and *S. aureus*) (Feng *et al.*, 2022). Septin recruitment to bacteria may be dictated by specific factors absent (or non-exposed) in *S. aureus*, which may also explain why septin caging of *L. monocytogenes* failed to be identified (Mostowy *et al.*, 2010). In the case of *S. flexneri* septin caging, LPS has been shown

to prevent septin binding to the bacterial surface, indicating that septins bind a component concealed in the *S. flexneri* cell envelope. *E.coli,* which is not bound by septins *in vitro*, is taxonomically indistinguishable from *S. flexneri* but does not express the virulence factor IcsA which is located at the pole of *S. flexneri*. Expression of IcsA significantly increased septin binding to *E. coli,* suggesting that IcsA disrupts LPS at the bacterial cell pole to expose a surface component recognised by septins (Lobato-Márquez *et al.*, 2021). Similarly, the Gram+ CW composed of diverse structural elements may prevent septin binding to a concealed component in the *S. aureus* envelope.

In summary, it may be challenging to characterize septin interactions with intracellular *S. aureus* due to the molecular complexity of host and bacterial cells.

'Bottom-up' approaches, such as cell-free *in vitro* reconstitution assays, based on purified proteins are an important strategy to investigate processes happening in a crowded cellular environment. Microscopy-based *in vitro* reconstitution assays have transformed our understanding of cytoskeletal components in infection biology, important examples include reconstitution of the *L. monocytogenes* actin tail (Loisel *et al.*, 1999) and reconstitution of vaccinia-based microtubule motility (Xu *et al.*, 2023). By using various substrates such as supported lipid bilayers or whole cell living bacteria, *in vitro* reconstitution assays based on purified recombinant septin complexes have significantly advanced our understanding of their properties and functions (Bridges and Gladfelter, 2016). Cell-free *in vitro* reconstitution of the *S. flexneri*-septin cage discovered that the bacterial surface protein IcsA promotes septin recruitment, while LPS prevented septin recruitment (Krokowski *et al.*, 2018).These findings highlight the power of a reductionist approach to investigate septin-bacteria interactions.

In the following **Chapter 4** we exploited our *in vitro* reconstitution system to investigate *S. aureus*-septin interactions, testing a variety of mutants affected in the expression of different surface factors.

Chapter 5. Bottom-up investigation of septin interactions with *S. aureus*

5.1. Introduction

5.1.1. Surface properties of *S. aureus*

S. aureus produces an arsenal of virulence factors that include toxins, immune modulatory factors and exoenzymes to promote immune evasion and maintain its intracellular lifestyle. Alongside these virulence factors, the bacterial CW and cell membrane are important components positioned at the host-pathogen interface to overcome the hostility of the host microenvironment (Silhavy *et al.*, 2010).

Unlike Gram- bacteria which have two cell membranes surrounding a thin layer of PG in between, Gram+ bacteria have a complex cell envelope made of a dense network of PG (30-100nm thick) surrounding a cytoplasmic membrane (**Figure 17**). Threading through the PG layers are long anionic polymers called teichoic acids (TAs), separated in wall teichoic acids (WTA) and lipoteichoic acids (LTA). Other important cell envelope components in Gram+ organisms include sortase A (SrtA)-CW-anchored surface proteins and capsular polysaccharides (CP), which are covalently attached to PG, membrane proteins and lipoproteins which are attached to the phospholipid membrane (Rajagopal and Walker, 2017).

To protect its cell envelope against antimicrobial compounds, such as antibiotics or cationic human antimicrobial peptides (CAMPs), the membrane sensor GraS regulates the multiple peptide resistance factor (MprF) and the DltABCD system to alter the surface positive charge upon exposure to antimicrobial peptides (AMPs) (Yang *et al.*, 2012). MprF and DltABCD activities promote an increased positive charge to the *S. aureus* surface envelope by

lysinylating membrane phosphatidylglycerol (for MprF) and alanylating teichoic acids (for DltABCD). An important component of Gram+ bacteria cell envelope is the PG layer which functions as a first line of defense against environmental insults and allows Gram+ bacteria to withstand the turgor pressure exerted on the plasma membrane (Monteiro *et al.*, 2019).

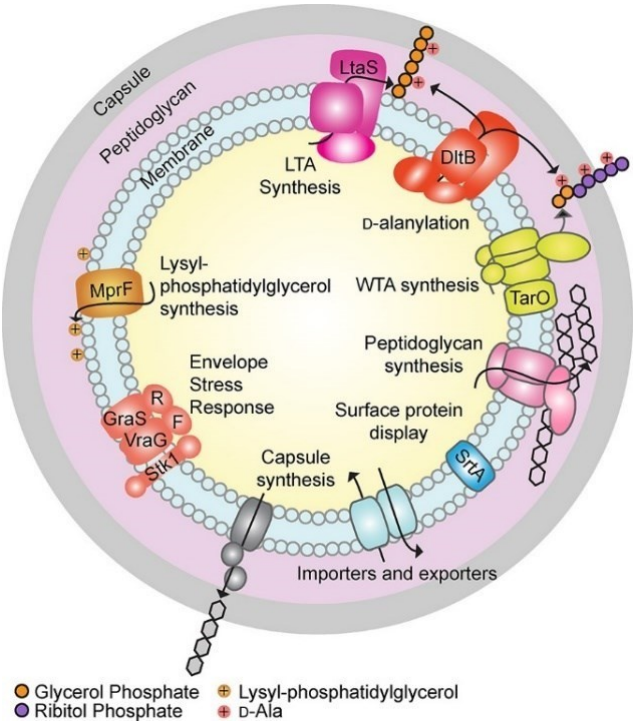


Figure 17. **Envelope structures of Gram+ bacteria**. Major pathways involved in the synthesis of the cell envelope include surface proteins, capsule, PG and TA synthesis. Tas can be modified by d-alanylation. Membrane charge can be modified by lysylphosphatidylglycerol synthesis. Adapted from Rajagopal and Walker, 2017.

5.1.2. Peptidoglycan: *S. aureus* armour and Achilles' heel

The PG layer is essential for viability and the biosynthetic pathway represents a target for AMPs and clinically used antibiotics since it is highly conserved across Gram+ organisms. AMPs and antibiotics can directly interact with PG precursors as well as the penicillin-binding proteins PBPs involved in the peptidoglycan biosynthesis pathway. Examples include mannopeptimycins, lantibiotics, defensins and glycopeptides antibiotics which bind to PG

precursors, as well as β-lactam antibiotics which interacts with PBPs (Malanovic and Lohner, 2016; Narchonai *et al.*, 2023).

PG is a biopolymer assembled from repeating disaccharide units cross-linked via peptide side chains (**Figure 18**).

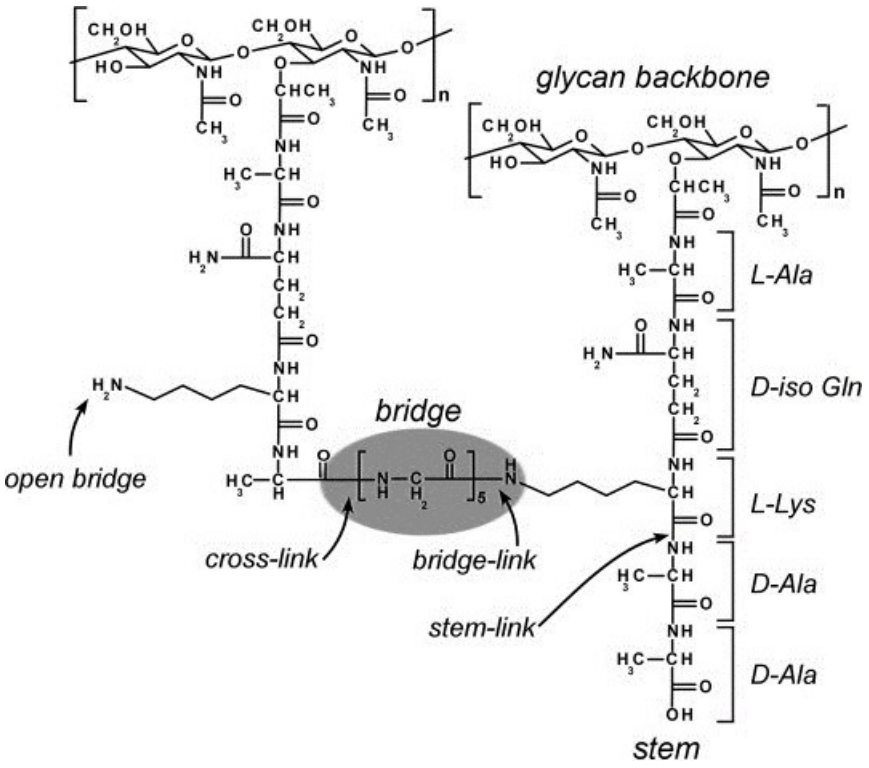


Figure 18. Chemical structure of the PG of *S. aureus*. PG repeat unit consists of disaccharide (glycan backbone), pentapeptide-stem, and bridge structure. Adapted from (Kim *et al.*, 2015).

In *S. aureus*, the average chain length of glycan strands is relatively short, averaging 6–18 disaccharide units and the disaccharide subunit consists of N-acetylglucosamine (NAG) coupled to N-acetylmuramic acid (NAM) (Vollmer *et al.*, 2008). Each NAM is associated with a linear peptide "stem" consisting of five amino acids whose composition varies from one organism to another. In *S. aureus*, the stem is formed by l-alanine, d-iso-glutamine, l-lysine, d-alanine and d-alanine (Perkins, 2012). The stems between glycan strands are connected through

a pentaglycine “bridge” extending from the third amino acid (l-lysine) of one of the stem peptides and the fourth amino-acid of another peptide stem (d-alanine). This high-level of cross-linking results in a strong 3D mesh-like layer.

PG biosynthesis is a highly conserved process that can be divided in 5 distinct stages : (1) precursor synthesis (stem pentapeptide attached UDP-N-acetyl muramic acid); (2) assembly lipid-linked monosaccharide Lipid I; (3) glycosylation of lipid I to form lipid II; (4) flipping of lipid II across the membrane and (5) polymerisation of cross-linked glycan strands by PG-binding proteins (PBPs) to give mature PG (Kumar *et al.*, 2022). PBPs have two major functions: through transpeptidases activity they crosslink PG units via the penta-glycine cross-bridge, while their glycosyltransferases activity performs crosslinking of two PG units through their sugar units. *S. aureus* possesses four core genome encoded PBPs (PBP1 to 4) and MRSA strains additionally possess the horizontally acquired PBP2a. Only PBP1 and PBP2 are essential for the final steps of PG biosynthesis and PBP3 has been shown to play a role in sidewall incorporation of PG in *S. aureus* to maintain a prolate spheroid cell shape (Reichmann *et al.*, 2019; Wacnik *et al.*, 2022). PBP4 is needed for the secondary cross-linking of PG, providing additional structural rigidity to the bacterial CW (Atilano *et al.*, 2010; da Costa *et al.*, 2018).

PG modifications (e.g attachment of teichoic acids, O-acetylation, N-glycosylation or de-N-acetylation) have been shown to provide resistance to lysozyme and linked to modulation of recognition of *S. aureus* PG by host receptors (Collins *et al.*, 2011). Using a *Drosophila* infection model, *S. aureus* autolysin Atl was shown to trim the outermost PG fragments that may extend beyond the TA layer to prevent PG recognition by host innate immune receptor named PGRP-SA (PG Recognition Protein-SA) (Atilano *et al.*, 2014). Similarly, a *Drosophila* infection model, WTAs have been shown to limit recognition of the PG layer by host PGRP-

SA, and WTAs are also involved in protecting PG against the hydrolytic activity of lysozyme *in vitro* (Atilano *et al.*, 2011).

In summary, *S. aureus* have a complex cell envelope (mediating host-pathogen interactions) composed of a thick PG shielded by surface proteins and other biomacromolecules. The requirement of PG for bacterial survival makes it an ideal target for clinically relevant antibacterial agents and host immune proteins (e.g., PGRP, lysozyme). It is thus essential to gain further insight into the biology of bacterial cell surfaces and how they are recognised by host cells.

Previously in **Chapter 4** we investigated septin interactions with intracellular *S. aureus*. Despite exploring a wide variety of experimental conditions, we were not able to identify clear septin association with intracellular *S. aureus.* In this **Chapter 5**, we exploited our cell-free *in vitro* reconstitution assay based on purified recombinant septin complexes to reduce complexity associated with the crowded cellular microenvironment. In particular, we aimed to test the hypothesis that (i) septins can interact with the Gram+ surface of *S. aureus* surface in our reductionist *in vitro* setting and (ii) *S. aureus* may express bacterial factors that inhibit septin recognition. Reconstitution of septin caging from purified proteins has been a powerful approach to study septin interactions with bacterial pathogens such as *S. flexneri* and *M. marinum* (Lobato-Márquez et al., 2021). We reasoned that a similar approach may help to identify key determinants of septin-*S. aureus* interactions that may have been masked by the complexities of the cellular environment.

5.2. Results

5.2.1. Purified septin complexes interact with *S. aureus* surface

While investigating septin interactions with intracellular *S. aureus*, in rare cases we observed clear septin cage entrapment of cytosolic *S. aureus,* reminiscent of the *S. flexneri*-septin cage (**Figure 10**). A previous study has shown that purified septins can bind to *S. flexneri* in the absence of any other host factors, and expression of a full length LPS layer restricted septin binding to bacterial surface (Lobato-Márquez *et al.*, 2021). Considering that it has been challenging to characterize septin-*S. aureus* interaction within the cellular context, we employed our *in vitro* assay to minimize complexity and assess direct interactions between purified septin complexes and the surface of *S. aureus*.

Native cell-isolated human septins exist as stable hexamers (composed of SEPT2-SEPT6-SEPT7) and octamers (composed of SEPT2-SEPT6-SEPT7-SEPT9) (Sellin *et al.*, 2011). Our *in vitro* reconstitution assay is based on purified recombinant septin hexamers SEPT2–SEPT6–SEPT7 because this hetero-oligomer was characterized previously and these three septins have been associated to *S. flexneri*-caging (Mostowy *et al.*, 2010; Sirianni *et al.*, 2016; Krokowski *et al.*, 2018). For fluorescence microscopy assays, a msGFP tag was fused to the N-terminus of SEPT6.

Septin polymerisation is highly dependent on ionic strength of the buffer where the reaction is occurring (Bertin *et al.*, 2008; Jiao *et al.*, 2020; Iv *et al.*, 2021; Fischer *et al.*, 2022). Electron microscopy data showed that at a low ionic strength (<150 mM monovalent salt), the minimal septins complexes self-assemble into filamentous structures. At higher ionic strength (>300 mM monovalent salt), septins mostly remain as individual complexes. Thus the *in vitro* reconstitution experiments were conducted in a minimal medium whose composition has been optimised to support both bacterial growth and septin complexes assembly into filaments (**Materials and Methods, section 2.9.5.**) (Lobato-Márquez *et al.*, 2021). Of note, overnight culture and exponential growth of bacteria used for *in vitro* assays are also realised in M9-based minimal buffer to ensure that (i) there are no residual traces of salt from the TSB that

would affect septin polymerisation, and (ii) to avoid switching *S. aureus* from a nutrient-rich TSB media to the minimal M9-based media.

Exponentially grown SH1000-mCherry were incubated with purified SEPT2-SEPT6-SEPT7 complexes for 2h. Then samples were washed and imaged by Airyscan confocal microscopy. Under these conditions, we observed septin association to the vast majority of live *S. aureus* SH1000-mCherry in a punctate pattern decorating the bacterial cell surface (92,6±5,0% of bacteria, n = 308, in biological triplicates) (**Figure 19**). However, we failed to observe cage-like structures covering most of the bacterial surface.

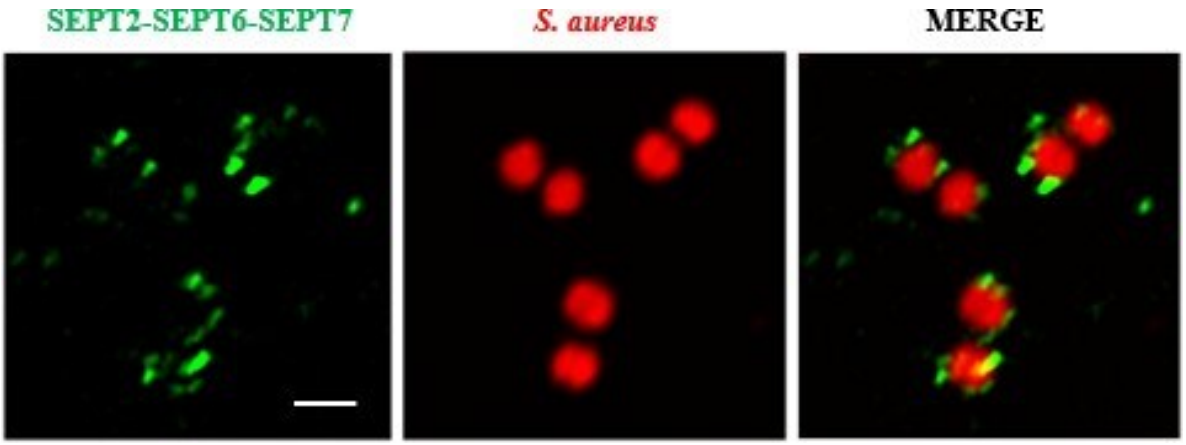


Figure 19. Purified septin complexes interact with *S. aureus* surface. Representative airyscan confocal image of septin-*S.aureus* SH1000-mCherry interactions *in vitro*. Purified septins are shown in green, *S. aureus* is shown in red. Scale bar, 1 μm

5.2.2. Septin binding to the *S. aureus* surface is stable over time

To study the stability of septin-*S. aureus* interactions and test whether we could observe an increase or loss of septin binding over time, *S. aureus* SH1000-mCherry was incubated with purified septin complexes for different time periods (ranging from 1 to 4h). Total fluorescence of purified septins bound to *S. aureus* was measured and no significant differences in septin binding was observed at any of the timepoints analysed, suggesting that septin binding to the

S. aureus surface *in vitro* happens as early as 1h and is stable over time (**Figure 20**).

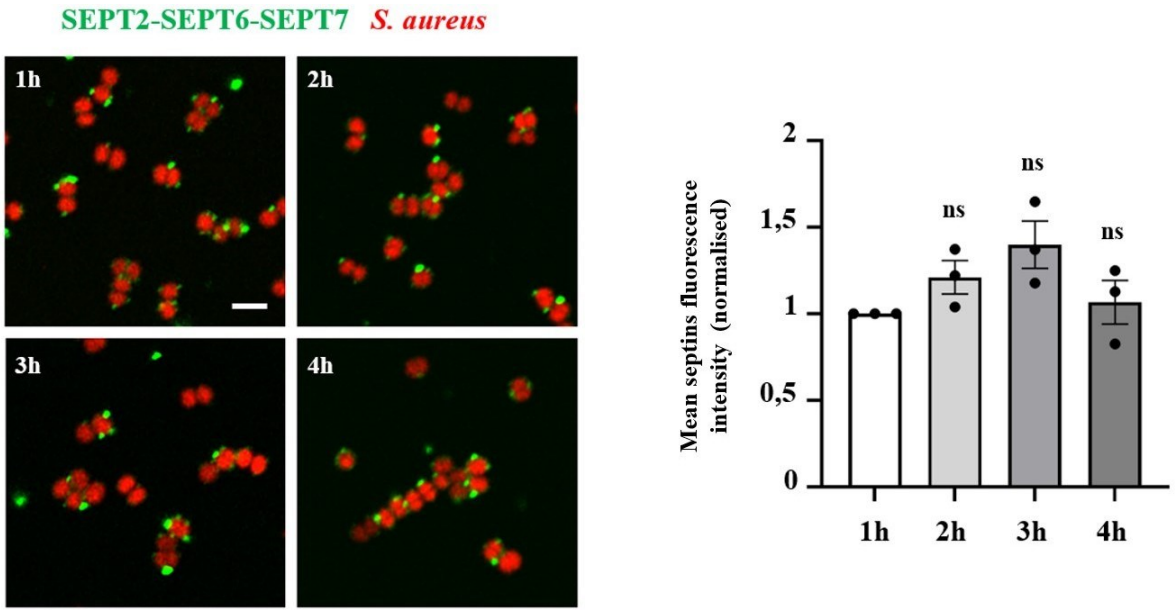


Figure 20. Septin binding to *S. aureus* surface is stable over time. A. Representative airyscan confocal image of septin-*S.aureus* SH1000-mCherry interactions *in vitro* after 1h, 2h, 3h and 4h incubation. Purified septins are shown in green, *S. aureus* is shown in red. Scale bar, 2 µm. **B.** Total fluorescence of purified septins bound to each bacterial cells from (A) was quantified. Data represent mean ± SEM normalised to t = 1h. For each time point, n > 500 bacteria in biological triplicates. N.s.; non-significant by 2-way Anova and Dunnett's post-test.

5.2.3. Bacterial growth stage does not affect septin binding

We next investigated the influence of the bacterial growth stage on septin binding by using *S. aureus* collected at exponential, early and late stationary stage (overnight culture). In this case,no significant differences were observed between each conditions (**Figure 21**). These results suggested that purified septins may be interacting with surface components that are constantly present throughout *S. aureus* growth.

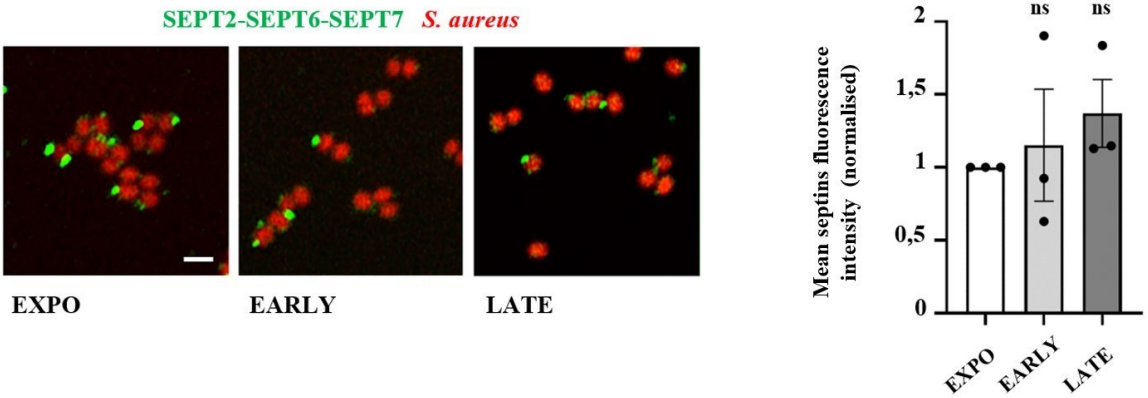


Figure 21. Bacteria growth stage does not affect septin binding. A. Representative airyscan confocal image of septin interactions with *S. aureus* SH1000-mCherry collected at exponential (left), early stationary (middle) and late stationary (right) growth stage. Purified septins are shown in green, *S. aureus* is shown in red. Scale bar, 2 μm. **B.** Total fluorescence of purified septins bound to each bacterial cells from (A) was quantified. Data represent mean ± SEM normalised to bacteria at exponential growth stage. For each condition, n > 500 bacteria in biological triplicates. N.s.; non-significant by 2-way Anova and Dunnett's post-test.

5.2.4. Wall teichoic acids prevent septin binding

In all conditions tested, septins bind to *S. aureus* SH1000-mCherry surface in a punctate-like pattern (**Figure 19**). In the case of *S. flexneri*, *in vitro* purified septins were mostly bound to one pole of *S. flexneri* WT, while *S. flexneri* Δ*rfaC* (which encodes a truncated LPS) is fully entrapped in septin cages (Lobato-Márquez *et al.*, 2021).

We reasoned that the punctate-like pattern of septin binding to *S. aureus* may be due to recognition of a target heterogeneously exposed on the bacterial surface. Alternatively, this heterogeneous binding pattern may be due to surface components restricting homogenous binding. An ideal candidate for this role is the highly-expressed surface-exposed WTA which constitutes the most abundant surface molecule in the *S. aureus* CW (Brown *et al.*, 2013). Tunicamycin is a highly selective inhibitor of TarO, the first enzyme in the WTA synthesis pathway (Campbell *et al.*, 2011). *S. aureus* were cultured overnight in the presence of 5μg/ml tunicamycin, and the subculture was exponentially grown in the presence of 5μg/ml tunicamycin to maximize depletion of WTAs from the CW. Pre-treated bacteria were incubated with purified septins, and after 2h the samples were analysed by microscopy. Strikingly, inhibition of WTAs synthesis led to a significant increase in septin binding to *S. aureus* (2.4 fold), with septins being recruited more homogeneously to the surface of tunicamycin-treated *S. aureus* (**Figure 22**).

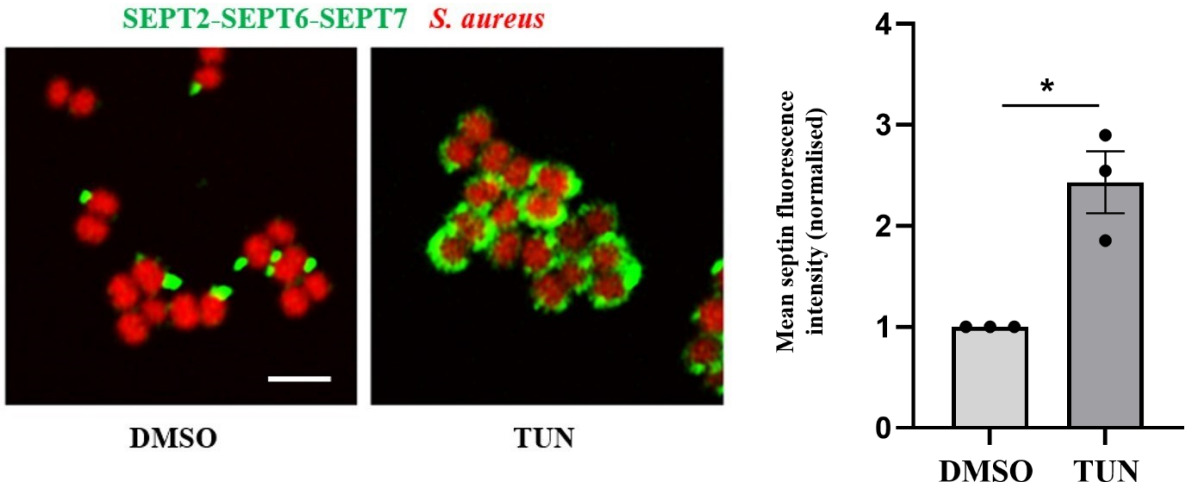


Figure 22. Wall teichoic acids prevent septin binding. A. Representative airyscan confocal image of septin interactions with *S. aureus* SH1000-mCherry pre-treated with DMSO (left) or with 5µg/ml tunicamycin (TUN) prior to septin incubation. Purified septins are shown in green, *S. aureus* is shown in red. Scale bar, 2 µm. **B.** Total fluorescence of purified septins bound to each bacterial cells from (A) was quantified. Data represent mean ± SEM normalised to bacteria pre-treated with DMSO. For each condition, n > 500 bacteria in biological triplicates. *, p-value <0.05 by two-tailed Student's t-test.

Additionally, similar to what was observed in **Figure 20** for untreated bacteria, total fluorescence of septins bound to tunicamycin-treated bacteria does not vary overtime, suggesting that septins are stably bound to the bacterial surface (**Figure 23**). However, because inhibitors can have off-target effects, testing a Δ*tarO* mutant is necessary to confirm the influence of WTAs on septin binding.

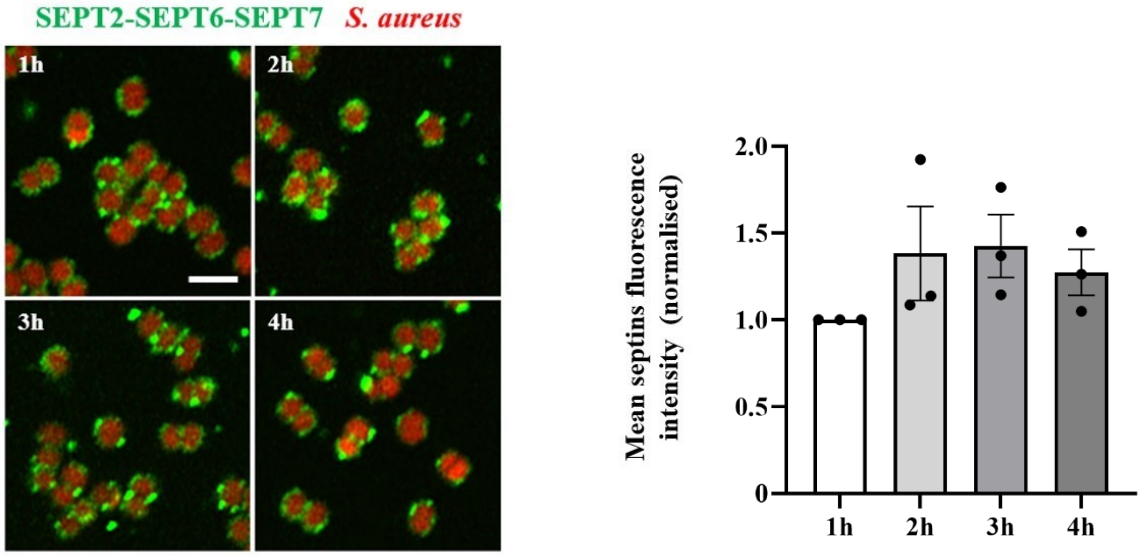


Figure 23. Septin binding to *S. aureus* surface depleted of WTA is stable over time. A. Representative airyscan confocal image of septin interactions *in vitro* with *S.aureus* SH1000-mCherry depleted of WTA after 1h, 2h, 3h and 4h incubation. Purified septins are shown in green, *S. aureus* is shown in red. Scale bar, 2 µm. **B.** Quantification of the total fluorescence of purified septins bound to *S. aureus* surface from (A). Data represent mean ± SEM normalised to t = 1h. For each time point, n > 500 bacteria in biological triplicates. N.s.; non-significant by 2-way Anova and Dunnett's post-test.

5.2.5. Identification of TarO, SrtA, Atl and MprF as regulators of septin binding

5.2.5.1. Characterisation of *S. aureus* mutants

To validate our results obtained with tunicamycin and search for other factors modulating septin binding to *S. aureus in vitro*, *S. aureus* Δ*tarO* and other mutants affected in the expression of key surface components were tested. We collaborated with the lab of Dr Pedro M. Pereira and Marianna G. Pinho from ITQB/NOVA (Institute of Chemical and Biological Technology António Xavier) in Portugal to perform screening using a collection of mutants from the Nebraska Transposon Mutant Library, a library made in *S. aureus* strain USA300 LAC JE2 (referred as *S. aureus* JE2 hereafter). Clean *S. aureus* mutants were then constructed to confirm results obtained using the transposon library. Characteristics of constructed mutants are described in **Table 5**.

Growth of the different strains in the minimal medium was also evaluated to ensure they could be used for *in vitro* assays (**Figure 24**). When considering the final time point (16h), there were no significant differences in the growth between WT and the different mutants, except for the Δ*atl* mutant. This can be explained by the fact that this mutant might be less subjective to the spontaneous bacterial autolysis which may happen throughout *S. aureus* growth (Bose *et al.*, 2012). It is also possible than the Δ*atl* mutant grow faster than the others, or that the OD is not reliable due to the clumping phenotype observed with this mutant.

Table 5. List of *S. aureus* JE2 strains used in this study.

Strains	Description	Source
JE2 wild type	Wild type strain, plasmid-cured derivative of MRSA USA300 LAC	P. Pereira lab
JE2 Δ*tarO*	JE2 *tarO* null mutant; *tarO* encodes for a glycosyltransferase required for the first step of WTA biosynthesis	P. Pereira lab
JE2 Δ*ltaA*	JE2 *ltaA* null mutant; *ltaA* encodes for a flippase that mediates the translocation of the glycolipid that anchors LTA to the cell membrane	P. Pereira lab
JE2 Δ*srtA*	JE2 srt*A* null mutant; srt*A* encodes for a transpeptidase that covalently bind proteins containing a CW sorting signal to the CW	P. Pereira lab
JE2 Δ*capE*	JE2 *capE* null mutant; *capE* encodes for the enzyme catalyzing the first steps in the synthesis of the soluble capsule precursor UDP-L-FucNAc.	P. Pereira lab
JE2 Δ*mprF*	JE2 *mprF* null mutant; *mprF* encodes for the enzyme catalyzing synthesis of the cationic phospholipid LysPG and its translocation to the outer membrane leaflet.	P. Pereira lab
JE2 Δ*atl*	JE2 *atl* null mutant; *atl* encodes for the major autolysin Major autolysin, a PG hydrolase with amidase and glucosaminidase domains.	P. Pereira lab
JE2 Δ*pbp4*	JE2 *pbp4* null mutant; *pbp4* encodes for a transpeptidase catalyzing peptide linkages between polymerized PG glycan chains	P. Pereira lab
JE2 Δ*oatA*	JE2 *oatA* null mutant; *oatA* encodes for enzyme catalyzing O-acetylation of PG	P. Pereira lab

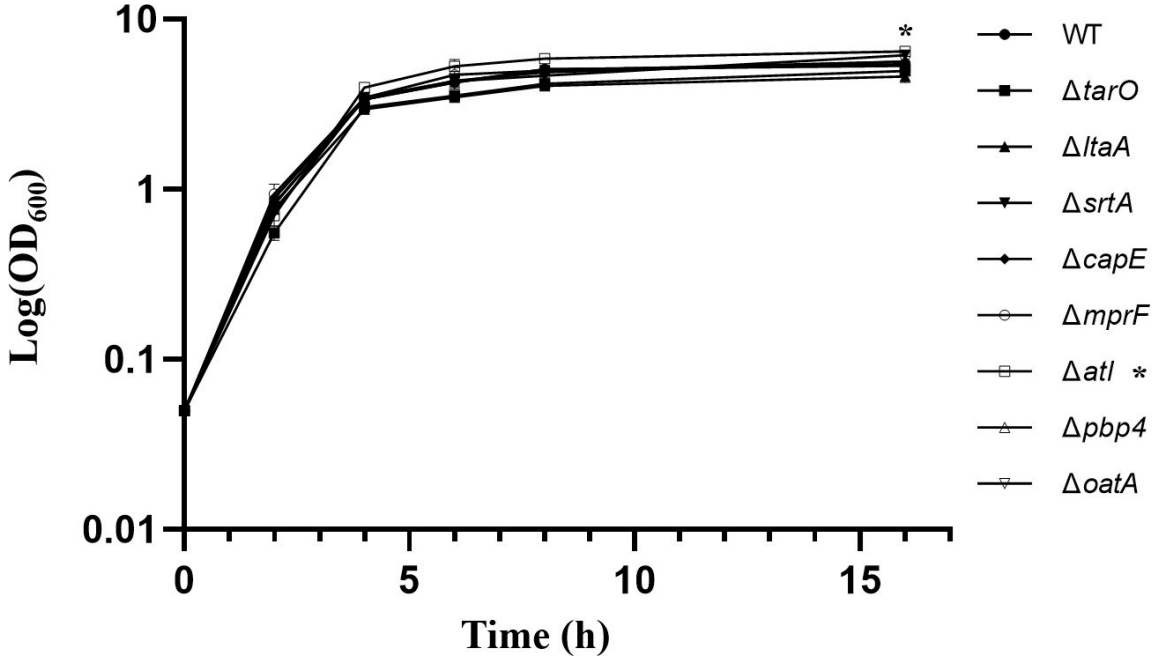


Figure 24. Growth of *S. aureus* JE2 WT and mutants in the minimal medium used for *in vitro* assays. Bacterial growth was determined by measuring the optical density (OD_{600}) represented in log_{10} scale at 2, 4, 6, 8 and 16h at 37°C with shaking. Cultures were started at an OD_{600} = 0.05. Graph represents measures done in biological triplicates. The final time point (16h) was considered for statistical analysis. *; p-value = 0.02 by 1-way Anova and Dunnett's post-test.

5.2.5.2. Screening to identify bacterial factors modulating septin recognition *in vitro*

We then tested for interactions between purified septins and the different mutants *in vitro*. We observed a significant increase (as compared to WT) in septin binding to Δ*tarO* (x2.4 fold), Δ*srtA* (x3.8 fold), Δ*atl* (x2.3 fold) and Δ*mprF* (x1.7 fold) (**Figure 25**). TagO is involved in synthesis of WTA, SrtA is involved in anchoring of surface protein in the CW, Atl encodes for the major autolysin involved in PG metabolism, while MprF synthetises positively charged lipids in the bacterial membrane promoting electrostatic repulsion of cationic antimicrobial peptides and antibiotics (**Table 5**). The results obtained for Δ*tarO* mutant (which do not produce WTA) are consistent with our findings that tunicamyin-treated *S. aureus* are more recognised by purified septins *in vitro*.

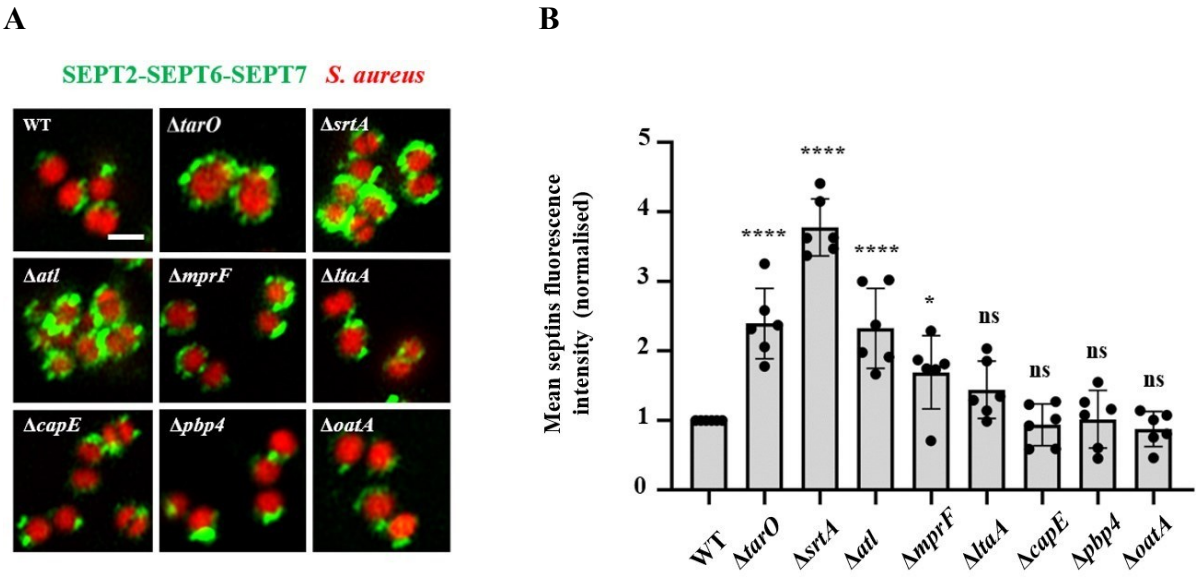


Figure 25. Increased septin binding to Δ*tarO*, Δ*srtA*, Δ*atl* and Δ*mprF* mutants. A. Representative airyscan confocal image of septin interactions *in vitro* with *S.aureus* JE2 WT and the indicated mutants. Purified septins are shown in green, *S. aureus* is shown in red. Scale bar, 1 µm. **B.** Quantification of the total fluorescence of purified septins bound to *S. aureus* surface from (A). Data represent mean ± SEM normalised to JE2 WT. For each condition, > 500 bacteria in biological triplicates. N.s., non-significant; ****, p-value <0.0001 by 2-way Anova and Dunnett's post-test.

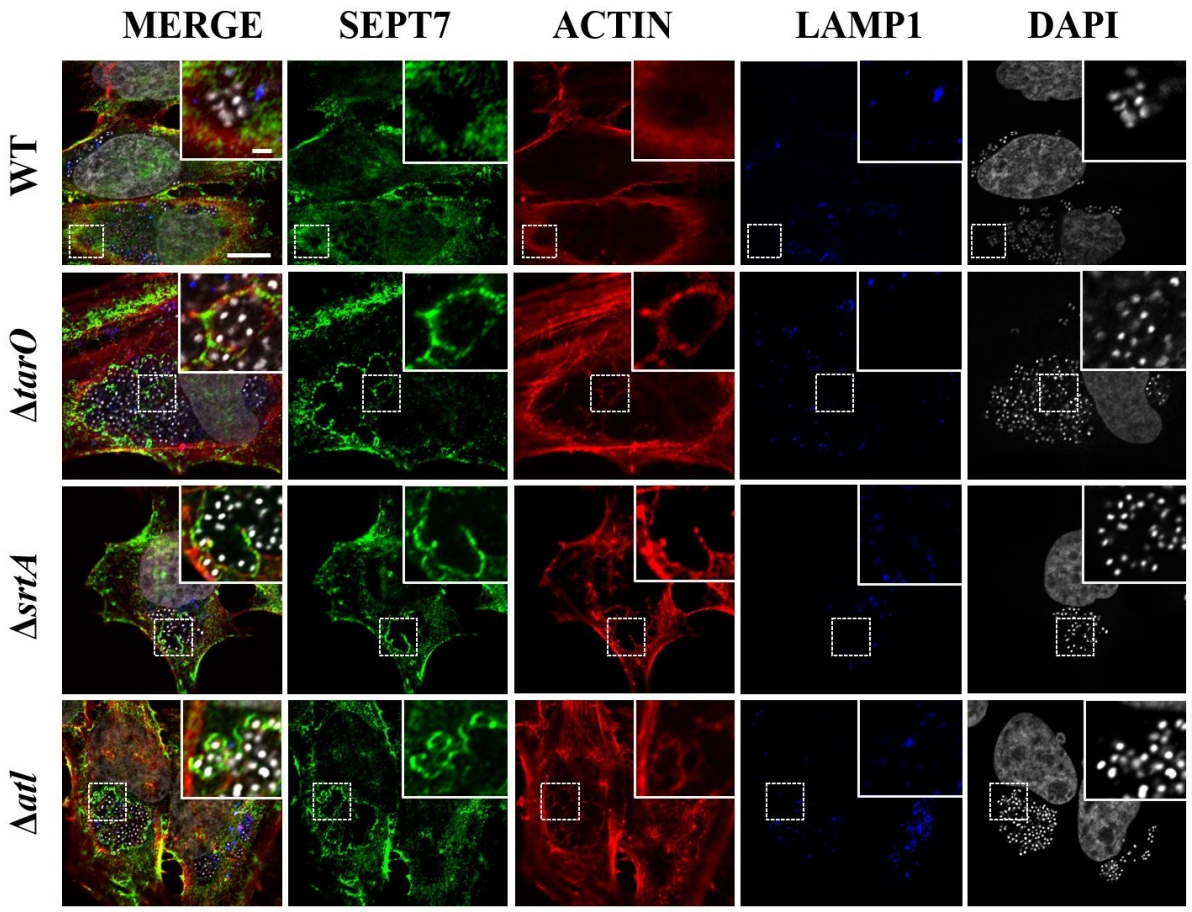
5.2.6. Testing of mutants identified by *in vitro* screening approach during HeLa cell infection

Reconstitution of septin caging from purified proteins has been a powerful approach to study septin interactions with bacterial pathogens *S. flexneri* and *M. marinum* (Lobato-Márquez *et al.*, 2021). Using this bottom-up strategy, we uncovered a role for TagO, SrtA, Atl and MprF in modulation of septin binding to the *S. aureus* surface. Considering that *in vitro* reconstitution cannot fully recapitulate processes happening in the complex intracellular microenvironment, we next performed HeLa cell infections to test if the mutants identified *in vitro* are also more recognised by endogenous septins *in situ* during infection of HeLa cells. We focused on Δ*tarO*, Δ*srtA* and Δ*atl* as these were the mutants showing the greatest increase in septin binding *in vitro*. HeLa cells were infected at an MOI of 1 with JE2 WT (as a control), Δ*tarO*, Δ*srtA* and Δ*atl* for 3h, fixed and labelled for SEPT7 and LAMP1 to identify bacteria located in lysosomal compartment or not. Due to their cell division defects as reported in (Vergara-Irigaray *et al.*, 2008; Chen *et al.*, 2013), those mutants formed large irregular clusters containing incompletely separated bacteria, which makes quantification of septins recruitment to individual bacteria challenging. To overcome this, we quantified the percentage of infected cells presenting septin-positive bacterial clumps. Similar to results obtained in **Chapter 4** with *S. aureus* SH1000-mCherry, RN6390-GFP, COL and MSSA PVL+, we could not observe any septin caging of JE2 WT (**Figure 26, top row**). In contrast, and as suggested by the *in vitro* reconstitution assay, we observed clear septins recruitment to the 3 differents mutants tested (**Figure 26**). These septin structures were similar to the structures described in **Figure 11**. Quantification showed that there was no significant difference between Δ*srtA* and Δ*atl (*~14 % and ~16% of cells infected displaying septin-positive bacterial clumps, respectively) whereas Δ*tarO* was significantly less recognised by septins than the 2 other mutants. None of the septin-positive

bacterial clumps were associated with the lysosomal marker LAMP1, highlighting that bacteria are not inside a lysosome and are likely cytosolic.

In the case of *S. flexneri*-septin caging, septins are well-known to associate with actin filaments (Mostowy *et al.*, 2010). Focusing on Δ*srtA* and Δ*atl* (for which we had the most events), we quantified the percentage of cells displaying both septin and actin positive clumps (as compared to the total number of infected cells displaying septin-positive bacterial clumps). We observed co-recruitment of septin and actin in 42 ± 16 % and 59 ± 12 % of the case for Δ*srtA* and Δ*atl* respectively, and the difference between the two was non-significant.

A



B

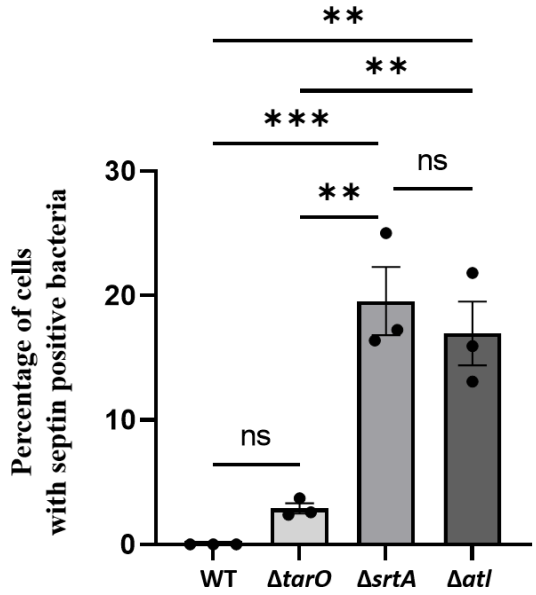


Figure 26. Septin recruitment to intracellular *S. aureus* JE2 Δ*tarO*, Δ*srtA* and Δ*atl* mutants. A. Representative airyscan confocal images of septin interactions with intracellular *S. aureus* JE2 WT, Δ*tarO*, Δ*srtA* and Δ*atl*. SEPT7 is shown in green, ACTIN is shown in red, LAMP1 is shown in blue and DAPI in white. Scale bar, 15 µm , inset 5 µm. **B.** Quantification of the percentage of infected cells presenting septin-positive bacterial clumps from total infected cells. For each condition, > 100 infected cells were quantified in biological triplicates. ns, non-significant; **, p-value <0.002; ***, p-value =0.0004 by 1-way Anova and Tukey's post-test.

5.2.7. Septins interact with purified *S. aureus* PG sacculi

WTAs and Atl activity have been shown to conceal *S. aureus* PG from detection by the PG recognition protein PGRP-SA (Atilano *et al.*, 2011; Atilano *et al.*, 2014). Whether JE2 Δ*srtA* mutant has a more exposed peptidoglycan is not known. This is possible because SrtA anchors a variety of surface proteins to peptidoglycan which could represent a physical barrier by causing steric hindrance for example. Considering that experiments (performed both *in vitro* and *in situ* during HeLa infection) showed that septin recruitment to Δ*tarO,* Δ*srtA* and Δ*atl* is increased as compared to the WT strain, we hypothesized that septins might sense the more accessible PG of *S. aureus*. To test whether purified septins directly interact with PG, we purified CW and PG sacculi (PG sacculi with WTAs) from *S. aureus* JE2 WT (as indicated in

Material and Methods 2.9.7.). Briefly, exponentially grown bacteria were boiled in hot SDS (to remove contaminating proteins and non-covalently bound lipoproteins), then mechanically broken and treated with nucleases (to remove nucleic acids), trypsin (to remove cell wall bound proteins), 8 M LiCl (to remove polypeptidic contamination), acetone (to remove membrane lipids and LTAs), washed with water and lyophilized. This lyophilized pellet containing peptidoglycan sacculi and covalently bound WTA was considered "purified CW fraction". Peptidoglycan sacculi were purified by extraction with cold hydrofluoric acid to remove WTA, washed and lyophilized to obtain the "purified PG fraction".

With these purified fractions, pull-down assays were performed where septins were incubated with purified CW and PG sacculi for 1h before centrifugation and washes. Due to their insolubility in aqueous solution, PG and CW will pellet after centrifugation and if septins interact with PG it will be retained in the PG pellet fraction. Purified septins precipitated with purified PG sacculi and CW, whereas in the control without PG only traces of protein were observed in the pellet, indicating that septins directly bind PG (**Figure 27A**). In parallel, after co-incubation of purified septins with purified sacculi, the sample was imaged by Airyscan confocal microscopy, and in this case, we observed clear septin association with purified sacculi. These preliminary results strongly suggest that septins are directly binding to PG (and not just co-pelleting with PG during the centrifugation step of the pull-down assay) (**Figure 27B**). In next steps, these pull-down assays will be repeated and septin interactions with other non-soluble oligosaccharides such as chitin, amylose and cellulose will be performed.

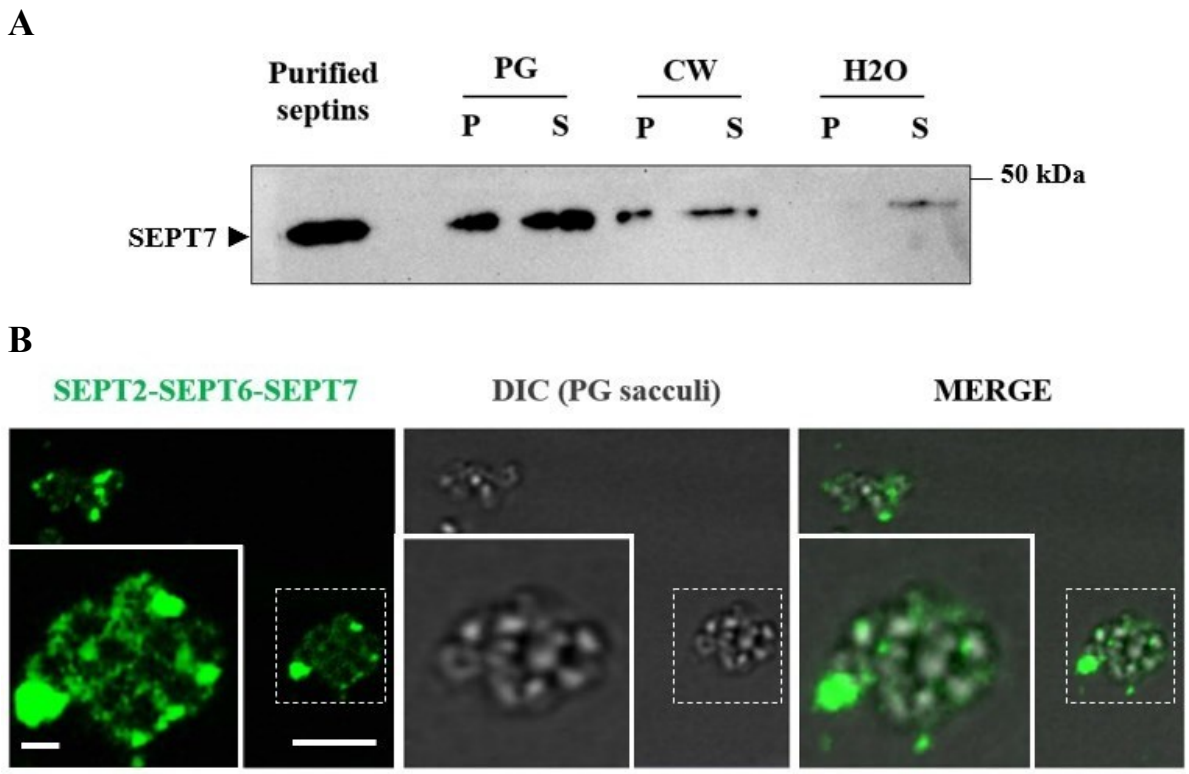


Figure 27. Septin binding to purified *S. aureus* PG sacculi and CW. A. Representative western blot of pull-down assays. Purified PG sacculi and CW fraction was incubated with purified septin complexes for 1h before centrifugation and washes. Unbound septins remained in the supernatant (S) upon centrifugation, while septins bound to the insoluble PG was co-precipitated and found in the pellet fraction (P). Purified septins only were loaded in the 1st lane as a control. **B.** Representative airyscan confocal image of septin interactions with purified *S.aureus* PG sacculi. Purified septins are shown in green, sacculi are visualised by DIC microscopy. Scale bar, 5 µm, inset 1 µm.

5.2.8. Discussion

S. aureus is increasingly recognized as an intracellular pathogen using the host cell as niche to promote its survival (Speziale and Pietrocola, 2020). In recent years, septins have emerged as key players in diverse microbial infection processes and a previous study from the lab indicated that septins could associate with intracellular *S. aureus* (Krokowski *et al.*, 2018; Robertin and Mostowy, 2020). In **Chapter 3** we highlighted a novel role for septins in *S. aureus* invasion, and in **Chapter 4** we investigated experimental conditions to explore septin interactions with intracellular *S. aureus*. Despite significant efforts, we were not able to observe

any clear septin recruitment to intracellular bacteria. In **Chapter 5**, we opted for a bottom-up cellular microbiology approach, using our cell-free *in vitro* reconstitution system based on purified septin complexes to study septin-*S. aureus* interactions. We showed that purified septins can directly interact with the WT *S. aureus* surface in a punctate pattern, and that this binding is stable overtime. Using tunicamycin to inhibit synthesis of WTA, one of the most abundant structures on *S. aureus* CW, we observed a significant increase of septin binding to the *S. aureus* surface. Further investigation using a selected subset of mutants affected in the expression of different surface components identified Δ*tarO* (synthesis of of WTA), Δ*srtA* (synthesis of CW anchored surface proteins), Δ*atl* (autolysis and PG metabolism) and Δ*mprF* (synthesis of positively charged lipid LysPG) as being significantly more recognised by septins as compared to WT cells. We tested our *in vitro* findings by performing infection of HeLa cells and showed that Δ*tarO*, ΔsrtA *and* Δ*atl* were also significantly more recognised than WT cells by endogenous septins *in situ*.

Septins have been shown to be involved at the early stages of autophagy in *S. cerevisiae* (Barve *et al.*, 2018). In the context of *S. flexneri* infection of human cells, septins are recruited to bacteria associated to autophagy markers *(*Krokowski and Mostowy, 2019; Sirianni et al., 2016). In the future, it will be important to investigate the fate of septin-caged entrapped *S. aureus* (*e.g.* are these bacteria targeted to autophagy?). Are septins pro- or anti-bacterial in the case of intracellular *S. aureus*? We can answer this question by depletion of septin expression by siRNA.

Septins have been associated with exocytosis of the ultra-large glycoprotein Von Willebrand factor in endothelial cells where they associate with the actomyosin machinery required for regulated secretion (El-Mansi *et al.*, 2023). In the case of *P. aeruginosa*, septin-caged bacteria were observed (albeit rarely) to be secreted out of infected cells (Aigal *et al.*, 2022). Although there are only few reports suggesting *S. aureus* interactions with the exocytosis pathway

(Rauch *et al.*, 2016), live-cell microscopy experiments of septin-caged *S. aureus* can be performed to assess an involvement of septins in *S. aureus* exocytosis.

Innate immune receptors sense conserved structures of microbes called pathogen-associated molecular patterns (PAMPs) (Janeway, 1989) including PG of both Gram+ and Gram- bacteria, an essential component of the bacterial CW and thus an excellent target of the immune system. Mammals have several PG recognition molecules including cell-surface CD14, Toll-like receptor 2 (TLR2), LRR- and pyrin domain-containing Protein 3 inflammasome (NLRP3) and intracellular nucleotide-binding oligomerization domain (Nod)-containing proteins Nod1 and Nod2 (Wolf and Underhill, 2018). In addition, mammals also express a family of PG recognition proteins named PGRPs with human PGLYRP1 being one of the most well described shown to bind to both Gram+ and Gram- bacteria and potentiate killing of intracellular bacteria by macrophages (Slonova *et al.*, 2020). *Drosophila* expresses PGRPs orthologs, including one called PGRP-SA which plays a key role in the recognition of *S. aureus*. Our results show that similar to what has been described for PGRP-SA (Atilano *et al.*, 2011; Atilano *et al.*, 2014), septin recognition of *S. aureus* Δ*tarO* and Δ*srtA in vitro* and *in situ* during HeLa cell infection is significantly increased. In a *Drosophila* infection model, WTAs and autolysin Atl activity are essential for concealing the PG of *S. aureus* and prevent its recognition by PGRP-SA. Moreover *S. aureus* SrtA heavily decorates the surface of PG with surface proteins which can potentially restrict exposure of PG similar to WTAs. This led us to investigate the interaction of septins with purified PG sacculi by performing pull down-assays. Consistent with our hypothesis, our results showed that septins can directly interact with purified PG sacculi.

These findings are of great interest and suggest many exciting research directions. Considering that protein sequences of SEPT2, SEPT6 or SEPT7 do not show any canonical sugar/PG binding domain, what septins domains are involved in PG binding? Can individual septins bind

to PG or do they need to be in complex or form filaments ? Can septins also bind Gram- bacteria PG *in vitro*, and which domain of PG is recognized by septins? Is septin-positive intracellular *S. aureus* also associated with other cell-autonomous immunity factors, transforming the bacterial surface into a signalling platform?

Further research is needed to fully understand the molecular mechanisms underlying septin-PG interactions, and how this discovery can help with infection control.

The Δ*srtA* mutant was also more recognised by septins in comparison to WT both *in vitro* and *in situ* during HeLa infection. Is the PG layer more exposed in this mutant (similarly to Δ*tarO* and Δ*atl*)? Although no studies to our knowledge have investigated PG exposure in the Δ*srtA* mutant, we speculate that PG could be more exposed given that SrtA anchors surface proteins to *S. aureus* PG. Alternatively, the PG structure of SrtA mutant may be altered, as described in the case of a Δ*tarO* mutant which highlighted a role for WTA as temporal and spatial regulators of PG metabolism (by regulating PBP4 to modulate PG cross-linking) (Atilano *et al.*, 2010). Additionally, enzymatic activity of some surface proteins can actively prevent septin binding to the *S. aureus* surface by cleavage or other modifications. Future work is required to examine how surface proteins prevent septin binding and whether PG is altered or exposed in such mutants.

Similarly, the Δ*mprF* mutant was also more recognised by septins *in vitro,* albeit less than Δ*tarO*, Δ*atl* and Δ*srtA* mutants. Further infections with HeLa cells are required to confirm whether this mutant is also more recognised by endogenous septins. MprF, whose activity decreases the net negative charge of bacterial surfaces, is known to play a crucial role in *S. aureus* virulence by promoting resistance to the AMPs and antibiotics (Assoni *et al.*, 2020). This suggests that septin recruitment to the *S. aureus* surface may also rely on electrostatic interactions across the cell envelope. Septins are recognised to interact with negatively charged

phospholipids via a conserved polybasic region close to the N terminus composed of 1–7 positively charged basic amino acids (Casamayor and Snyder, 2003; Tanaka-Takiguchi *et al.*, 2009). Future investigations could explore the involvement of this polybasic region for septin-*S.aureus* interactions.

Moreover, a recent study highlighted a role of MprF in modulation of LTA biosynthesis in *Bacillus subtilis* and *S. aureus*, and that LTA length and abundance regulate cellular level and activity of the major autolysin in the cell wall. It will be interesting to test the level and activity of SrtA and TagO in an MprF mutant, and also test if their PG is more exposed.

In conclusion, the findings of this chapter provide valuable insights about the mechanisms underlying septin interactions with intracellular *S. aureus*. To test the role of these interactions *in vivo*, future work using animal models such as the *S. aureus*-zebrafish infection model establish in our laboratory could be exploited (Ulhuq *et al.*, 2020).

6. Perspectives and future work

6.1. Highlights

Investigation of host cytoskeleton during bacterial infection has led to discoveries fundamental to both cell and infection biology. Septins, the 4th component of the cytoskeleton, are involved in different stages of the bacterial infection process. In particular, septins play key roles in cell-autonomous immunity by entrapment of intracellular *S. flexneri* (a Gram- pathogen). The process of septin cage entrapment has also been observed in the case of infection by *P. aeruginosa* (another Gram- pathogen), and preliminary data suggested that septins could interact with the Gram+ pathogen *S. aureus*. *S. aureus* is a global health threat due to its high virulence and propensity to develop drug resistance. Initially considered as an extracellular pathogen, its intracellular lifestyle is now acknowledged as a key aspect of its pathogenicity to escape killing by the host immune system and antimicrobial compounds. Thus, understanding mechanisms underlying the internalisation and intracellular survival of *S. aureus* is essential for development of effective antimicrobial therapies.

For this thesis we investigated septin interactions with *S. aureus*. Our main findings are summarised in **Figure 28**.

Our main findings are :

- Septins are recruited with actin and phosphorylated myosin light chain II to *S. aureus* invasion sites engaging integrin α5β1 (Chapter 3);
- SEPT2 and SEPT7 depletion reduces *S. aureus* invasion into host cells and the percentage of bacteria associated to actin, integrin α5β1 and activated FA kinase (FAK) (Chapter 3);
- SEPT2 depletion increases surface expression of integrin α5, *S. aureus* adhesion and protein levels of integrin α5, β1 and FAK (Chapter 3);

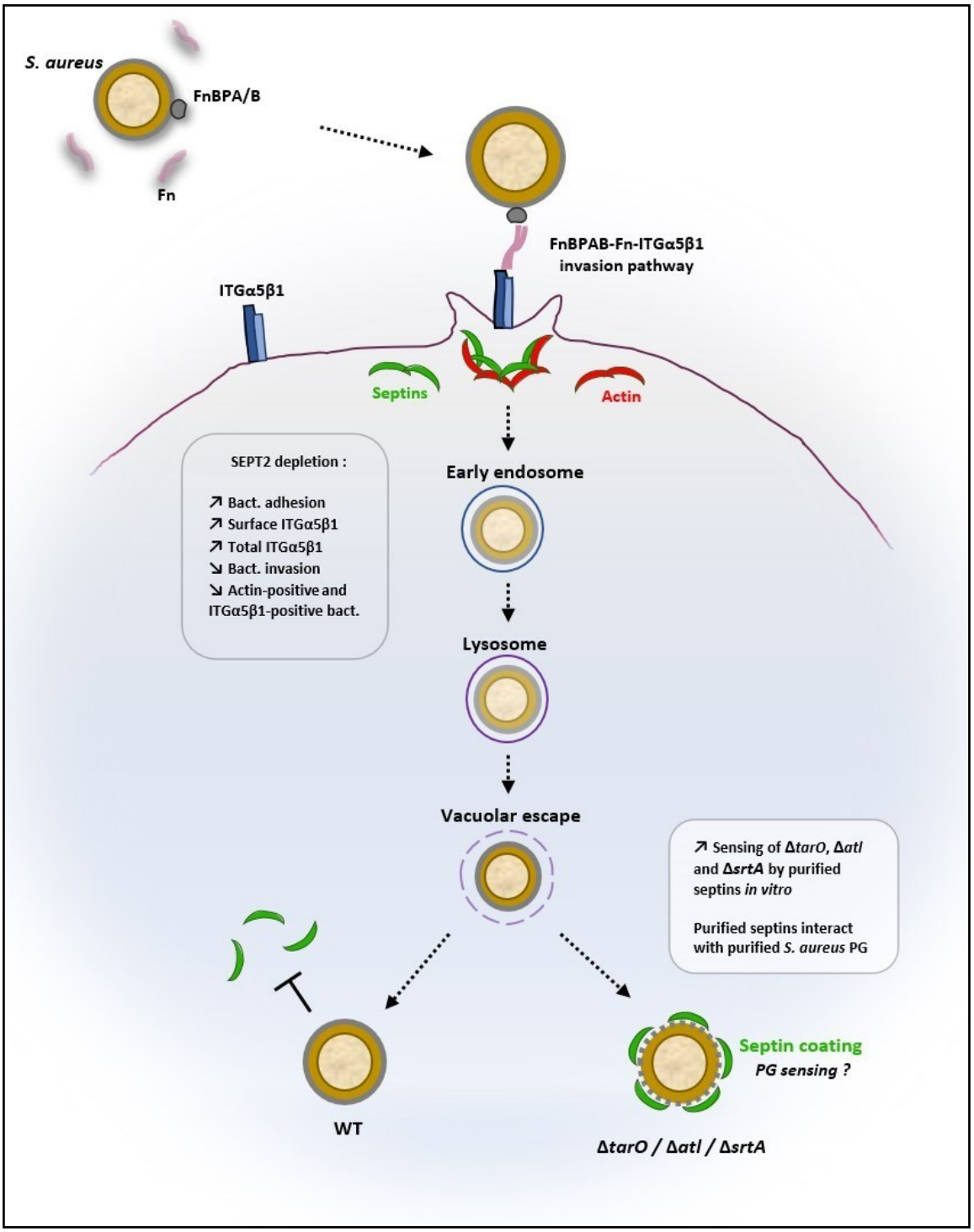


Figure 28. Proposed model of septin interations with *S. aureus* during infection of HeLa cell.

The results of **Chapter 3** highlight a novel role for septin in integrin α5β1-mediated invasion which can potentially be extended to other clinically relevant pathogens engaging this pathway.

- Septins rarely interact with intracellular *S. aureus* WT (Chapter 4);
- Experimental parameters (such as MOI, growth stage, growth medium) do not affect septin interactions with intracellular *S. aureus* WT under the experimental conditions tested here (Chapter 4);
- Other experimental parameters (such as bacterial strain, host cell type) do not affect septin interactions with intracellular *S. aureus* WT under the experimental conditions tested here (Chapter 4);

The results of **Chapter 4** hinted at how characteristics of *S. aureus* CW or its virulence factors may actively evade septin recognition under our experimental conditions.

- *In vitro* reconstitution based on purified septin proteins showed that septins can interact with the surface of *S. aureus* WT in the absence of other host factors (Chapter 5);
- Screening of mutants revealed WTA (Δ*tarO*), CW anchored surface proteins (Δ*srtA*), PG metabolism (Δ*atl*) and positively charged lipid LysPG (Δ*mprF*) as factors preventing septin binding to the *S. aureus* surface *in vitro* (Chapter 5);
- HeLa cell infections showed that mutants Δ*tarO*, Δ*srtA* and Δ*atl* are significantly more recognised than *S. aureus* WT by endogenous septins, and that septins can bind to purified *S. aureus* PG sacculi (Chapter 5);

The results of **Chapter 5** illustrate how our bottom-up *in vitro* reconstitution approach can be used to identify factors modulating septin recognition of *S. aureus* surface, and in this case uncover septins as a novel sensor of bacterial PG.

6.2. Regulation of integrin α5β1-mediated *S. aureus* cellular invasion by the septin cytoskeleton

In **Chapter 3** we revealed that septins play key roles in the invasion of *S. aureus* into host cells. We showed that septins are recruited as collar-like structures with actin at the invasion site, similar to what is observed for bacterial pathogens *S. flexneri* and *L. monocytogenes* (Mostowy *et al.*, 2009), highlighting a fundamental role for septins in bacterial invasion. In the case of *S. aureus*, septin recruitment is triggered by activation of the FnBPAB-Fn-integrin α5β1 entry pathway. In agreement, we showed that coating of beads with purified GST-FnBPA is sufficient to promote septin recruitment at the invasion site.

Previous *in vitro* work (using purified septins and silica beads of different diameter) showed that septins sense membrane curvature at the micron-scale (Bridges *et al.*, 2016). In this case, septins were shown to be maximally recruited to 1 µm sized beads, with less recruitment to beads of increasing size (3, 5, 6.5 µm). Considering their membrane curvature-sensing ability, we hypothesized that membrane curvature presented by the *S. aureus* phagocytic cup may promote septin recruitment. In preliminary work, we tested for recruitment of septins and integrin to GST-FnBPA-coated beads of 1 and 3 µm. In the case of 3µm GST-FnBPA beads, quantifications showed a non-significant decrease in septin recruitment as compared to 1µm GST-FnBPA beads (34% vs 44%, $p = 0.19$) **(Figure 29 A and B)**. Considering that differences were not significant, we cannot yet conclude on the association of septin / integrin to beads of different sizes at this stage. In the future, to test whether septins recruitment to integrin α5β1-engaging structures also depends on micrometer-scale topographical features of the curved plasma membrane, GST-FNBPA coated ellipsoidal beads (Buckley *et al.*, 2020) and microfabricated pillar substrate of diverse shape (Gupta *et al.*, 2015) could be used.

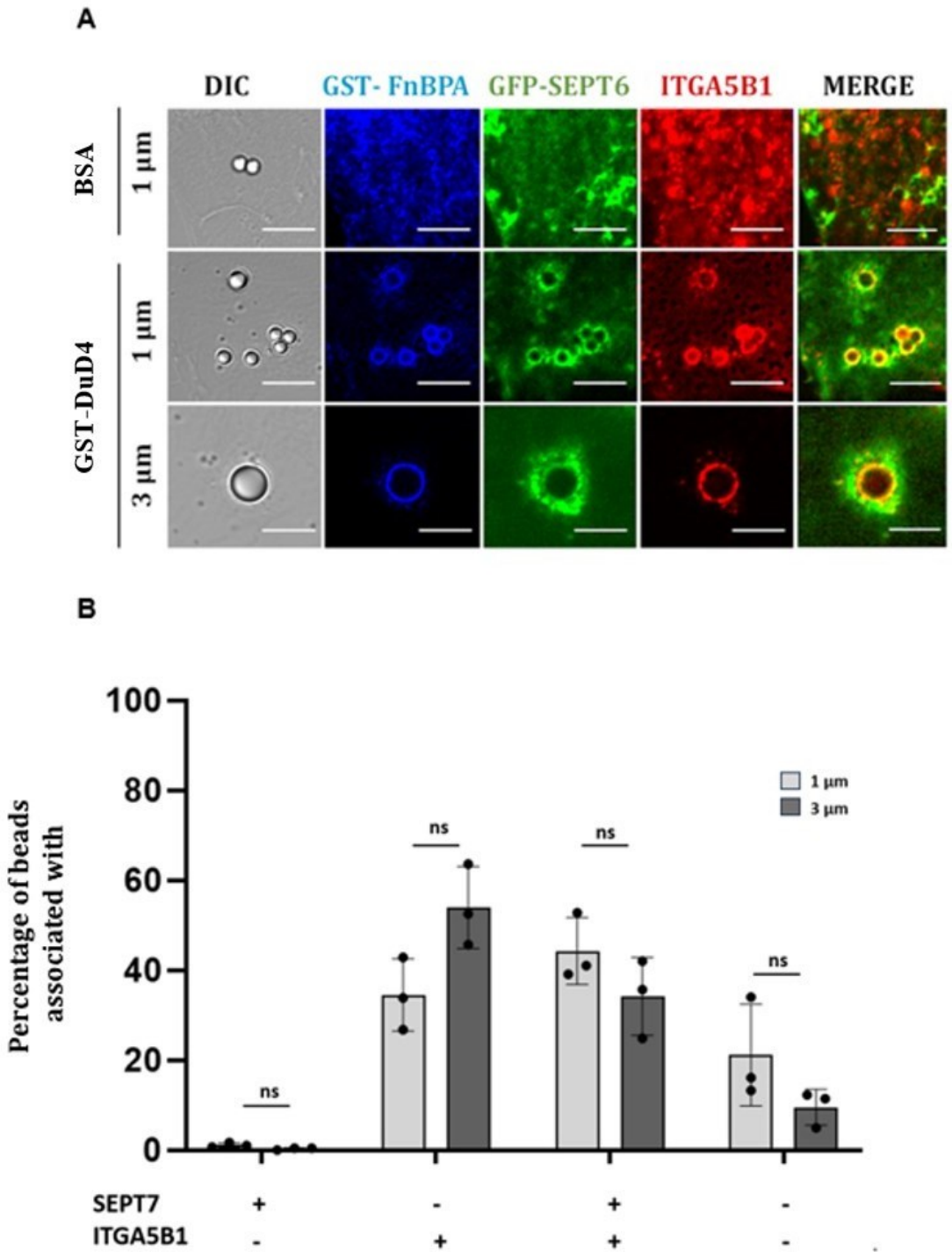


Figure 29. Septins recruitment to GST-DuD4 particles of different sizes. A HeLa cells stably producing GFP-SEPT6 were challenged with GST-DuD4-coated beads of 1 and 3 µm diameter for 15 min in DMEM + 10% FBS. After fixation, cells were stained for GST (blue) and active integrin α5β1 (ITG α5β1, red). DIC microscopy is shown to visualize the beads. Scale bar represents 2 µm. **B.** HeLa cells were challenged with GST-DuD4 coated beads of 1 and 3 µm diameter for 15 min in DMEM + 10% FBS. The graph represents mean percentage ± SEM of beads localizing with SEPT7 and/or active integrin α5β1 (ITG A5B1) from (A). n = 647 beads (1 µm diameter beads) and n = 621 (3 µm diameter beads) from three independent experiments. ns: non-significant by two-tailed Student's t-test.

FAs are integrin rich signalling platforms linking the ECM to the cytoskeleton. Septins have been shown to be involved in FA maturation via its interactions with the actomyosin network (Dolat *et al.*, 2014). However, in contrast to the *S. aureus* infection context where septins are recruited at integrin α5β1-rich invasion sites, septins are typically excluded from FAs and are on distal ends of actin stress fibers that contact FAs (Martins *et al.*, 2023). Physical properties of the ECM (i.e.,three-dimensional 3D vs two-dimensional 2D substrates) have been shown to influence the protein make-up of adhesion structures. An example are the 3D-matrix adhesions which differ from focal and fibrillar adhesions characterized on 2D substrates in their recruitment of integrin α5β1, paxillin and other cytoskeletal components (Cukierman *et al.*, 2001). Considering *S. aureus* or beads topography, the cell may sense them differently which trigger recruitment of proteins which may not be observed when integrin binds fibronectin on a 2D substrate. In preliminary work, we showed that, similar to coating of latex beads with GST-DuD4, coating of latex beads with purified fibronectin only triggers integrin activation and septin recruitment (**Figure 30**), highlighting the 3D property of the substrate as a signal for septin recruitment to activated integrin α5β1-rich cell area. During active internalisation of particles vs sensing of fibronectin on flat surface, cellular signalling may be different due to the extensive membrane remodelling and mechanical signal of membrane tension.

Future experiments could explore whether this phenotype is observed in more mechanically active cells that dynamically remodel the ECM. Similarly, septins recruitment to integrin α5β1-rich cell area could also be investigated during cell migration in 3D matrices and more complex physiologically relevant environments.

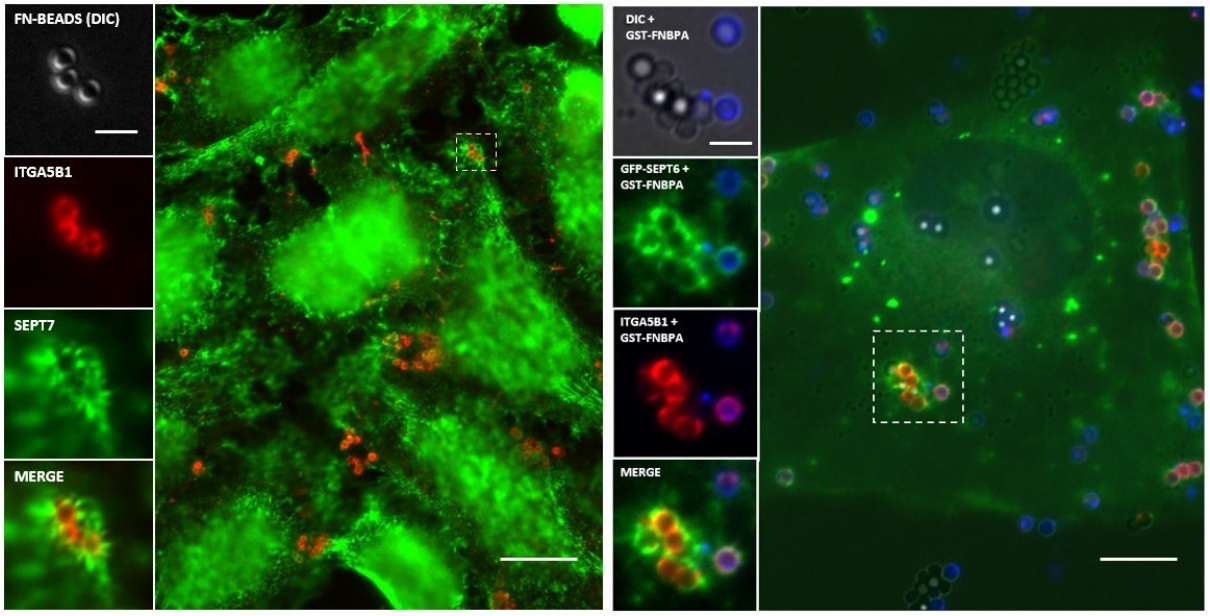


Figure 30. Fibronectin coating on 3D particle is sufficient to trigger septin recruitment. A. HeLa cells were challenged with Fn-coated beads of 1 µm diameter for 15 min in DMEM. After fixation, cells were stained for SEPT7 (green) and active integrin α5β1 (ITGA5B1, red). Fn-coated beads are shown in the DIC channel. **B.** HeLa cells were challenged with both Fn-coated and GST-DuD4 coatedbeads of 1 µm diameter for 15 min in DMEM + 10% FBS. After fixation, cells were stained for GST (blue) and active integrin α5β1 (ITGA5B1, red). Fn-coated beads are shown in the DIC channel only whereas GST-FnBPA appear in both DIC and are positive for GST (blue).

During the last decade, it has become clear that integrin α5β1 is much more than simply adhesion molecules, as they are now considered as a significant player in various host-pathogen interactions involving clinically relevant bacteria, fungi, and viruses (Ulanova *et al.*, 2009). Can a better understanding of integrin α5β1 and FA biology help with design of therapeutic strategies against infection? A role for autophagy via proteins such as LC3 and Neighbor of BRCA1 gene 1 protein (NBR1) in FA turnover has been described (Kenific *et al.*, 2016). Moreover, internalized β1-integrin were shown to become progressively recruited on LC3B-positive autophagy-related endomembranes (Barrow-McGee *et al.*, 2016). More recently, using *Streptococcus pneumoniae* which also express FnBP to activate integrin, it was shown that integrin α5β1 triggers autophagy, thus linking integrin α5β1 signalling to host innate immunity against invading FnBP expressing pathogens (Wang *et al.*, 2020). The septin

cytoskeleton has been linked to canonical autophagy as well as antibacterial autophagy (Mostowy and Shenoy, 2015). In the future, it will be interesting to test whether integrin α5β1-initiated autophagy is a common host response for host defense against invading pathogens, and whether septins play a role in this mechanism.

6.3. Investigating septin interactions with intracellular *S. aureus*

Septins have been shown to interact with various intracellular pathogens, such as vaccinia virus where septins trap virions on the plasma membrane to suppress their release, or *C. trachomatis* where septins arrange F-actin fibers on the bacterial inclusion to promote bacterial dissemination (Pfanzelter *et al.*, 2018; Robertin and Mostowy, 2020). In the case of the Gram- bacteria *S. flexneri*, septins form a cage around intracellular bacteria, targeting them to lysosomal degradation. Preliminary data suggested that septins could also entrap intracellular *S. aureus*, a Gram+ pathogen whose cell envelope differs drastically from that of *S. flexneri*.

In **Chapter 4** we aimed to investigate septin interactions with intracellular *S. aureus* . However identifying septin caged intracellular *S. aureus* was particularly challenging as these events were rarely observed. We performed a variety of assays where we changed a variety of experimental parameters, such as MOI, bacterial strains or host cell types to test if this would impact septin caging of *S. aureus*. Despite significant efforts, results mostly suggested that septins do not interact with intracellular *S. aureus* in our experimental settings. At this stage we concluded that either septins do not interact with *S. aureus* or that a *S. aureus* secreted / expressed a surface factor that actively preventing septin binding. Similarly, to GBPs which specifically target Gram- bacteria, septin recognition of intracellular bacteria could also be

specific to Gram- bacteria (such as *S. flexneri* and *P. aeruginosa*). (Feng *et al.*, 2022).

Additionally in this study we investigated septin-*S.aureus* interactions using NPPCs, will results be similar for professional immune cells such as macrophages ? Does the same scenario hold true in an *in vivo* context ? Future studies could gain valuable insights by employing different cell types or whole animal models to investigate septin-*S. aureus* interactions. Within the host organism, *S. aureus* may encounter a variety of stressors (e,g, AMPs, factors produced by competing microbiota) that have potential to damage its cell envelope or interfere with other cell wall biosynthetic pathways, thereby influencing bacterial and host response.

From these results, we concluded that it might be challenging to characterize septin interactions with intracellular *S. aureus*. The *in vitro* reconstitution of the *S. flexneri*-septin cage led to the identification of bacterial factors promoting and restricting septin cage entrapement (Lobato-Márquez *et al.*, 2021). We hypothesised that this same reductionist bottom-up approach may help to provide insights into septin-*S. aureus* interactions that could not be easily discovered using infection of tissue culture cells.

6.4. Bottom-up investigation of septin interactions with *S. aureus*

In **Chapter 5**, we employed our *in vitro* reconstitution system based on purified septin complexes to study septin-*S. aureus* interactions. We showed that purified septins interact with the *S. aureus* surface in a punctate pattern and that this binding is stable over time. This heterogenous binding pattern was reminiscent of the heterogenous binding of septin to *S. flexneri* WT surface, where only polar recruitment could be observed. Because the LPS layer

was preventing homogenous septin binding to WT *S. flexneri* surface, we hypothesized that a component of the *S. aureus* cell surface may also inhibit septin binding.

WTAs are long polymers which are the most abundant component of the *S. aureus* cell envelope, involved in resistance to host recognition and antimicrobial molecules (Guo *et al.*, 2021). Tunicamycin is a potent and highly selective inhibitor of TarO commonly used to impair WTAs synthesis (Maria *et al.*, 2014; Zhu *et al.*, 2018). Tunicamycin treatment of *S. aureus* significantly enhanced septin binding to the bacterial surface, resulting in homogenous binding of the bacterial surface. These results suggested that WTAs prevent septin recognition of a component buried in the CW. To gain further insights into factors modulating septin recognition of *S. aureus,* we performed a screen of selected mutants affected in expression of diverse surface components. This enabled us to confirm a role for WTAs (Δ*tarO*) and identify surface proteins (Δ*srtA)*, positively charged lipid LysPG (Δ*mprF*) and activity of autolysin (Δ*atl)* as factors preventing septin recognition of *S. aureus* surface *in vitro.* That both Δ*tarO* and Δ*atl* are more recognised by septins (as compared to WT bacterial cells) was highly reminiscent of the specificity of the PG binding protein PGRP-SA which binds more to exposed PG of Δ*tarO* and Δ*atl* mutants (Atilano *et al.*, 2011; Atilano *et al.*, 2014). This prompted us to test whether septins may also recognise *S. aureus* PG. In agreement, pull-down assays using purified *S. aureus* PG sacculi demonstrated that septins could interact directly with PG.

There is a possibility that because of their ability of forming filamentous structures, septins may be physically entangled with the non-soluble PG sacculi, resulting in non-specific interactions. Pull-down assays with other non-soluble oligosaccharides such as chitin, cellulose and amylose will be of great interest. Ultimately, biochemical experiments with superior sensitivity would have to be performed to identify whether septins binds to soluble peptidoglycan fragments and which fragments are bound (*e.g.* by surface plasmon resonance).

We conclude that septins are a novel sensor for bacterial PG. As a result, many new questions emerge. It is next of great interest to validate and understand how septins can recognise PG, and how this differs from other canonical sensors of PG. In the absence of canonical sugar/PG binding domains, what septin domain(s) can recognise PG? Human SEPT6 possess an amphipathic helix domain that senses micron-scale membrane curvature to promote *S. flexneri* septin caging (Lobato-Márquez *et al.*, 2021). In future work, our *in vitro* reconstitution system using can be used to test the role of other septin domains such as the GTP-binding domain, C-terminal coiled-coil and septin unique element (SUE) domain. Similarly, we could also test mutations known to prevent septin polymerisation into filaments (asking whether septin filamentation is required for cage entrapment of *S. aureus*).

Does septin association to intracellular *S. aureus* also promote recruitment of other cell-autonomous immunity proteins? PG sensing proteins (including members of the NLR family) can recruit a variety of effector molecules, such as pro-caspases, to promote NF-κB activation and inflammatory processes. Further biochemical and structural work is needed to understand the molecular mechanisms underlying septin-PG interactions in more detail.

6.5. Summary

This thesis demonstrated that using *S. aureus* can help to discover new functions and properties of the septin cytoskeleton in host-pathogen interactions. In **Chapter 3** we described for the first time a role for septins in the integrin α5β1-mediated invasion of host cells by *S. aureus*. In **Chapter 4** we performed a variety of experiments to suggest that intracellular *S. aureus* WT prevented septin recognition of its surface. In **Chapter 5** we identified key surface factors modulating septin recognition of the *S. aureus* surface using our cell-free *in vitro* reconstitution system, highlighting PG (an essential component of *S. aureus* CW) as a novel target of septin recognition. In the future, *in vivo* studies will be required to test the role of septins in *S. aureus*

infection at the whole animal level, to understand how manipulation of septin functions can be used to fight infection and guide host-directed therapeutic strategies. Given the emerging role of septins as a key player in different host-pathogen interactions, there is potential to integrate our *in vitro* reconstitution system into an automated fluorescent imaging platform and screen for different intracellular bacterial pathogens and mutants to help understand the key determinants of septins-bacteria interactions.

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